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Pravastatin[™] Suppresses Venous Shear Stress Dependent Induction of Aquaporin 1 Protein Expression in Human Umbilical Vein Endothelial Cells in vitro

Raphael J. Crum

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**PravastatinTM Suppresses Venous
Shear Stress Dependent Induction
of Aquaporin 1 Protein Expression
in Human Umbilical Vein
Endothelial Cells *in vitro***



Honors Thesis

Raphael J. Crum

Department: Biology

Advisor: Carissa M. Krane, Ph.D.

April 2017

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Abstract

Background: Aquaporins (AQPs) are transmembrane water channels that facilitate osmotically-driven transmembrane water flux. AQP1 is abundantly expressed in vascular endothelium, where it functions in endothelial cell migration, wound healing, and cell volume regulation in response to mechanical stimuli. AQP1 protein abundance is enhanced in cultured human umbilical vein endothelial cells (HUVECs) in response to fluid shear stress. Statins, a pharmacological class of HMG-CoA reductase inhibitors, suppress the early development of vein graft intimal hyperplasia by improving endothelial cell function, most likely by affecting endothelial response to arterial shear stress. Statins may also differentially regulate the expression of specific AQPs by altering their abundance and/or subcellular localization. Therefore, it is hypothesized that AQP1 may function as a component of the mechanosensory complex in endothelial cells and therefore is subject to shear stress-induced gene regulation and modulation of expression by statins.

Methods: HUVECs were cultured in microfluidic chambers under static (0 dynes/cm²) and venous fluid shear stress (6 dynes/cm²) in the presence or absence of 5 μ M Pravastatin™. Fluorescent immunocytochemistry was used to detect AQP1 protein expression. Images were captured using confocal laser scanning microscopy. Mean fluorescent intensity (in arbitrary units) was determined using ImageJ software and normalized to control conditions (time zero, static culture with no Pravastatin™).

Twenty-five to thirty cells from two independent experiments were analyzed for each experimental condition.

Results: AQP1 expression increased 1.4-fold ($p < 0.05$) after 24 hours, and 1.5-fold ($p < 0.05$) after 48 hours in cells cultured under venous fluid shear stress as compared to the control. This shear stress-induced expression was blocked by incubation with PravastatinTM and reduced by 27% ($p < 0.05$; control vs. venous shear stress with PravastatinTM). PravastatinTM had no effect on AQP1 expression in static cultures ($p > 0.05$ vs. control). These data indicate that PravastatinTM suppresses shear stress dependent induction of AQP1 in cultured HUVECs.

Conclusions: Results show that the shear stress-dependent induction of AQP1 protein expression is suppressed by PravastatinTM. Results further demonstrate a potential function of statin drugs in regulating the expression of AQPs that is both independent of their cholesterol-lowering function and related to the regulation of AQP1 in vascular endothelial cells.

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Chapter 1: Literature Review

1.1 The Role of Water in Human Physiology

Water (H_2O) is an essential constituent of life. Water is a small tetrahedral shaped molecule with two hydrogen atoms, one oxygen atom, and four valence electrons around oxygen. The electronegative properties of the oxygen atom and the four valence electrons around oxygen lend to the polar nature of H_2O . This electronegative differential between the more negative oxygen atom of the molecule and the two more positive hydrogen atoms creates a permanent polar dipole that results in a partial negative charge on the oxygen atom and a shared partial positive charge on the two hydrogen atoms. The polar properties of water allow for water to serve as a universal solvent for polar and charged molecules. This polar nature leads to the distinct electrostatic, cohesive, and adhesive properties of H_2O (36). As a universal solvent for polar and charge molecules, H_2O is the fundamental constituent of the various fluid filled components of the human body. Water makes up approximately 60%-90% of human body weight and is an essential component of normal cellular function, chemical and metabolic reactions, transport of nutrients, thermoregulation of body temperature, and the elimination of waste from the body (50).

Given these chemical properties, H_2O plays a fundamental role in biological systems as a universal solvent. The polarity of the H_2O molecule provides an environment in which a large number of solutes are readily soluble in H_2O . These solutes include salts and other electrically charged or polar compounds. Water serves an additional thermodynamic role in the solubility of molecules that are not polar or electrically charged. In this instance, H_2O forms a unique hydration shell that surrounds the “dissolved” solute and effectively increases the entropy of the cellular system (36).

Combined, the diverse capabilities of H₂O as a solvent for polar and nonpolar molecules provide the foundational environment for the necessary biochemical and metabolic reactions that are present at the foundation of cellular and organismal health and function.

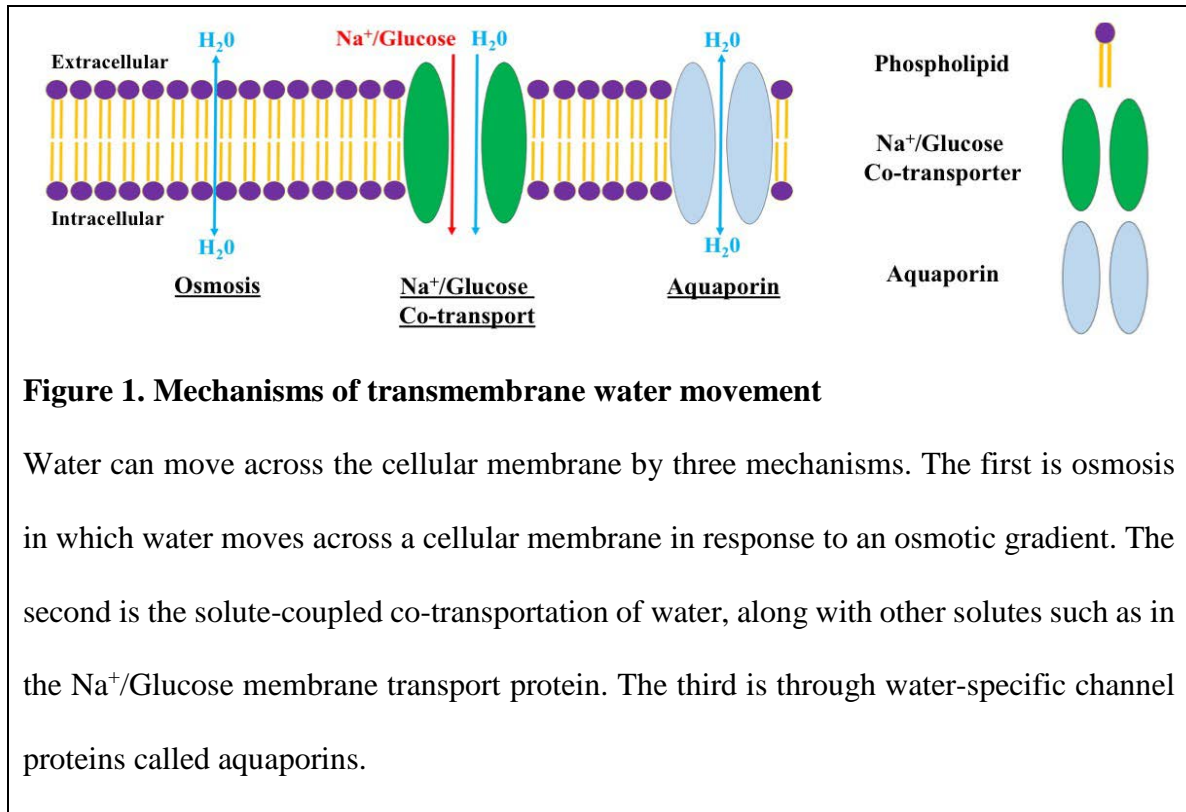
1.2 Osmolarity and Water Regulation at the Organismal and Cellular Level

Given the necessity of H₂O and hydration in human physiology, it is important to understand how H₂O balance and homeostasis in the human body are managed. The fundamental principle of fluid balance surrounds managing the sensitive osmolarity of the intracellular and extracellular fluids. Osmolarity is a measure of the concentration of the fluid compartments of the human body expressed as a total number of solute particles per liter of solution (48). Osmoregulation is a vital process that regulates osmolarity and osmotic pressure in a cell by means of regulating the composition and concentration of intracellular and extracellular fluids (2).

At the organismal level in human physiology, osmoregulation is achieved through integrated mechanisms involving the hypothalamus, pituitary gland, and the kidneys (44). Circumventricular organs (CVOs) located in the hypothalamus and existing outside of the blood-brain barrier contain specialized cells called osmoreceptors that respond to changes in blood osmolarity. The osmoreceptors that are located specifically in the organum vasculosum of the lamina terminalis in the brain send neural projections to the supraoptic nucleus of the hypothalamus that promotes the release of vasopressin (also known as anti-diuretic hormone) from the posterior pituitary. This process ultimately targets the kidneys to adjust H₂O reabsorption and excretion to compensate for increases or decreases in osmolarity of the extracellular fluid (11). As a result, H₂O balance at the

organismal level is controlled through water retention and urine excretion from the kidneys.

At the cellular level in human physiology, H_2O is distributed between three fluid-filled compartments in the body: the intracellular fluid, the interstitial fluid, and the blood plasma (48). Water balance between the intracellular and extracellular compartments is mediated through three fundamental mechanisms. The first is osmosis, a slow and non-selective mechanism driven by osmotic gradients. The second is solute-coupled co-transport, a mechanism by which water essentially “hitches a ride” with another solute in a co-transport mechanism. For example, H_2O can enter a cell via this mechanism by means of the sodium/glucose co-transporter through which sodium and glucose are co-transported into the cell with a “hitch-hiking” H_2O molecule (7). The third mechanism is one by which H_2O balance is regulated at the cellular level through channel proteins in the membrane that form pores in the cellular membrane that allow for the passive, regulated, and selective route for the transmembrane flux of H_2O molecules. These channel proteins are called aquaporins (AQPs) (1, 46) (Fig. 1).



1.3 Aquaporins

While osmosis and solute-coupled water transport are effective mechanisms for transporting water in and out of cells, these processes are limited. Osmosis is slow, non-selective for water, and is driven only by osmotic gradients. Solute-coupled transport requires a solute to function, and it is a non-direct and non-selective mechanism for water transport. These limitations are compensated for with AQPs. Aquaporins are regulated and selective proteins that provide a route through the cell membrane for osmotically-driven transmembrane water flux. In the compartmentalized system of a cell, a selectively-permeable membrane exists that is composed of a bilayer of phospholipids. This phospholipid bilayer has externally and internally facing phosphate groups that are hydrophilic (“water-loving”) and interact with the polar H₂O component of the

extracellular and intracellular fluid. The parts of the bilayer that are inside the membrane are composed of long-chain carbohydrate lipids that are non-polar and lipophilic/hydrophobic (“lipid-loving”/ “water-fearing”). The non-polar nature of these membrane lipids significantly hinders the passive and spontaneous migration of H₂O through the membrane. The solution to this issue is the presence of membrane spanning water channels called AQPs that create a functional pore in the cellular membrane by which H₂O can pass through the membrane.

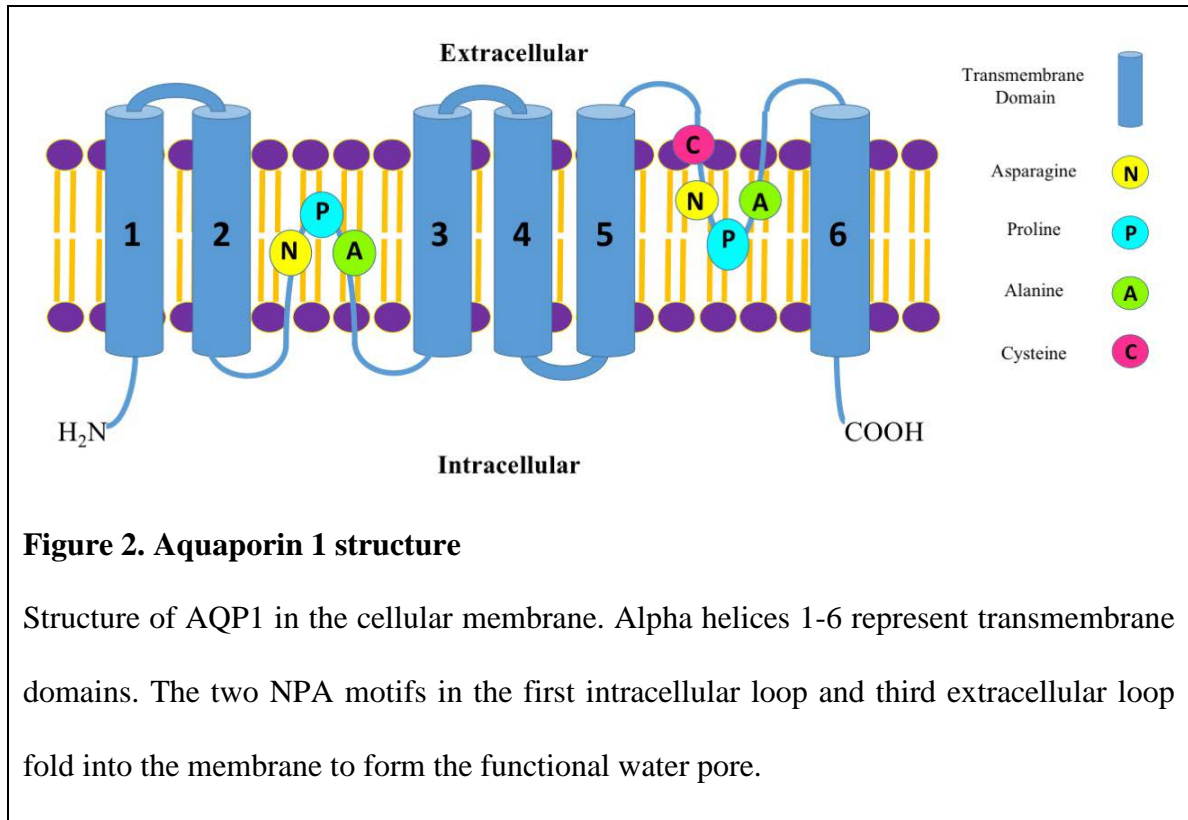
Aquaporins were discovered by experimental serendipity. In 1991, Dr. Peter Agre and colleagues were investigating the group of Rh antigens expressed on the surface of red blood cells. During this investigation, the group consistently observed an unusual protein band on gel electrophoresis experiments. At first the researchers assumed this band of 28 kilodaltons (kDa) size was a contaminant in early protein preparations (45). To the group’s surprise, this unusual band was an unidentified protein that is highly abundant in human red blood cells and the epithelial membranes of the proximal renal tubules in the kidney (46). While the actual function of this protein was unknown to Agre and his colleagues, they hypothesized that this protein might be responsible for H₂O transport across the cellular membrane due to the high permeability of H₂O in red blood cells and in the cells of proximal renal tubules.

With this hypothesis in mind, Agre and colleagues used oocytes from the African clawed frog, *Xenopus laevis*, and expressed this unknown protein in these cells by microinjecting *in vitro* transcribed CHIP28 RNA into the oocytes. Since these cells normally have a low cellular permeability to H₂O, the researchers were interested in investigating if the exogenous expression of this proposed water channel in the oocytes

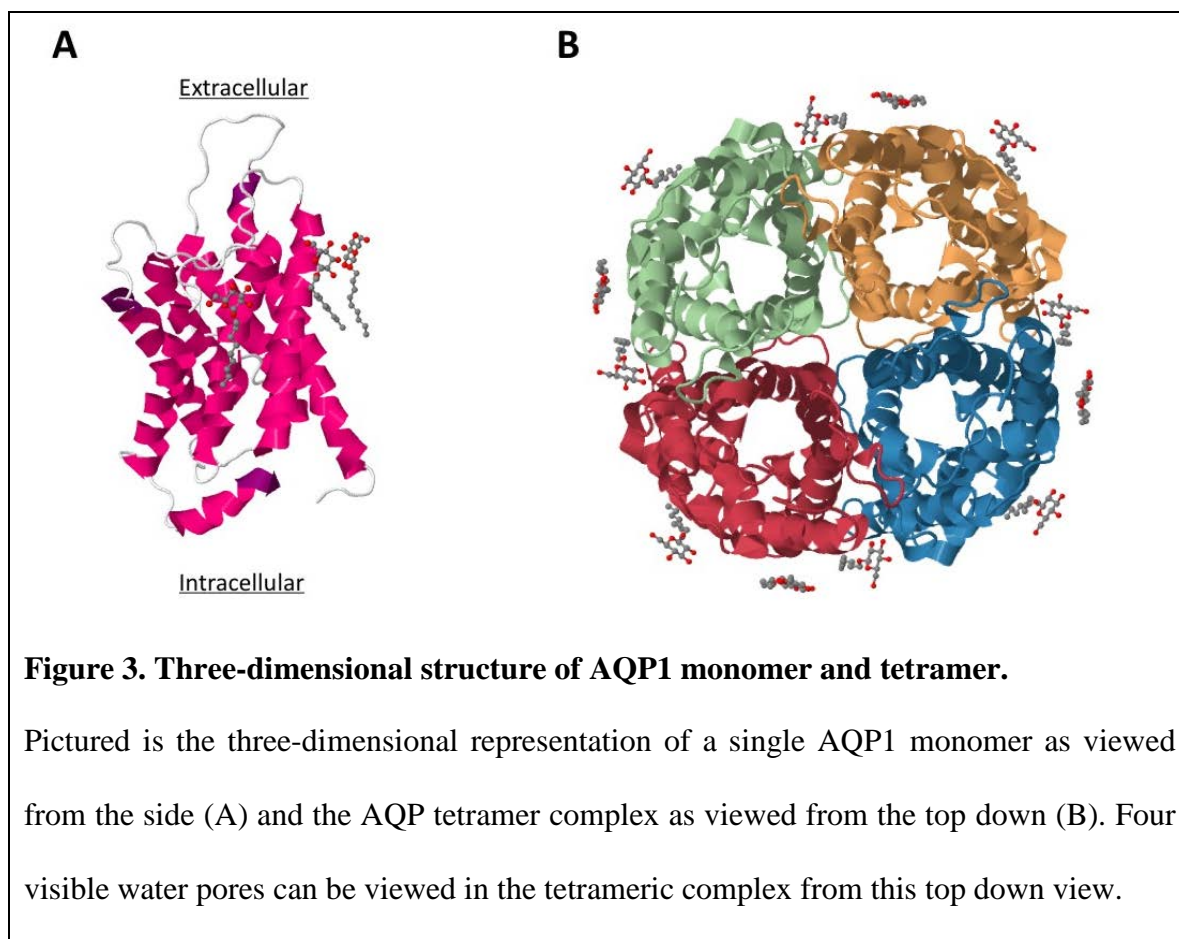
would alter the H₂O permeability of these cells. As expected from the group's hypothesis, the expression of this 28 kDa protein in the oocytes dramatically increased the water permeability of these cells (46). Agre later discovered that this protein existed in two forms: a non-glycosylated form at 28 kDa in size and a glycosylated form at 60 kDa (46). In 2003, Agre was awarded the Nobel Prize in Chemistry for the discovery of CHIP28 (channel-like integral protein of 28-kDa) (46), today referred to as Aquaporin 1 (AQP1). Since the discovery of AQP1, other researchers have identified 13 human AQP proteins (AQP0-AQP12) (13, 19, 20, 54).

1.4 Structure and Function of Aquaporins

AQPs belong to the major intrinsic proteins (MIP) family of transmembrane proteins (1). From high-resolution x-ray crystal diffraction, it has been determined that AQPs exist as a tetramer of four AQP protein monomers. Four AQP monomers assume a quaternary protein structure in the cellular membrane (4). Each monomer is composed of six membrane-spanning helical domains with two intra- and extracellular loops that form the functional water pore of the aquaporin (54). All AQPs, with the exception of AQP7, -11, and -12, contain a conserved three amino acid domain of Asparagine (N), Proline (P), and Alanine (A) within these loops (22, 56) (Fig. 2). In AQP7, the first intracellular loop is an AAA motif and the second extracellular loop is a NP and Serine (S) motif (56). In AQP11, the first intracellular loop is NP and Cysteine (C), and in AQP12 the first intracellular loop is NP and Threonine (T) (13, 20).



The narrow pore region of the AQP is formed through the interaction of the amino acids located within the two intermembrane loops and the transmembrane domains (His 182, Arg 197, Cys 191, Phe 58). These interactions form a pore size of 2.8-Å (52) (Fig. 3).



Structural analysis and simulations of the molecular dynamics of these water channel proteins have determined that H₂O moves through these channels in a single-file manner by which only one H₂O molecule is transported through the channel at a time. The H₂O molecule travels through the channel by electrostatically and sterically interacting with the intermembrane amino-acid residues of the tetramer and by forming and breaking short-lasting hydrogen bonds between the H₂O molecules and the amino acid residues of the channel (51).

Through the unique structure and function of AQPs, these channel proteins serve to facilitate osmotically driven transmembrane H₂O flux (35). Cells that express AQPs in their cellular membranes have on average a ~5 to ~50-fold increase in osmotic H₂O

permeability as compared to cells that do not express AQPs in their cellular membranes (30). The 13 mammalian AQPs are expressed in diverse cells and tissues throughout the human body. The cells that express AQPs in these cells and tissues have a high capacity for H₂O permeation. Listed below in Table 1 is the tissue distribution of the 13 AQPs (13, 19, 20, 54).

Table 1: Location of aquaporin isoforms in the human body

Aquaporin Isoform	Location in the Human Body
0	Lens
1	Blood Vessels, Kidney Proximal Tubules, Eye, Red Blood Cells, and Ear
2	Kidney Collecting Duct (principal cells)
3	Kidney Collecting Duct (principal cells), Epidermis, Urinary, Respiratory, and Digestive Tracts
4	Brain Astrocytes, Eye, Ear, Skeletal Muscle, Stomach Parietal Cells, and Kidney Collecting Ducts
5	Salivary, Lacrimal, and Sweat Glands. Lungs.
6	Intracellular Vesicles in the Kidney Collecting Ducts
7	Adipocytes, Testis, and Kidney
8	Kidney, Testis, and Liver
9	Liver and Leukocytes
10	Intestine
11	Adipocyte, Kidney, Liver
12	Pancreas

Of the 13 isoforms of AQPs, AQP1 is specifically expressed in microvascular endothelium located in areas outside the brain such as in the microvessels of the kidney, lung and airways, secretory glands, skeletal muscle, pleura, and peritoneum (6, 8, 10, 14, 16, 37). In the vascular endothelium, AQP1 functions to facilitate osmotically-driven transmembrane H₂O flux, wound healing and angiogenesis, as well as in the pathophysiology of fibrosis in selective disease states (18, 53).

1.5 Coronary Artery Disease: Clinical Presentation, Disease Pathophysiology, and Treatment.

Coronary Artery Disease (CAD) is the leading cause of death in American men and women. Indeed, 1 in 5 deaths in American men and women result from CAD (26). On a global scale, it is anticipated that the mortality caused by CAD will increase to around 23.4 million a year by the year 2030 (49). CAD ultimately manifests itself after at least one of the epicardial arteries of the heart becomes congested with a buildup of cholesterol and lipid plaques (Fig. 4).

Gross Anatomy of the Heart

Anterior view

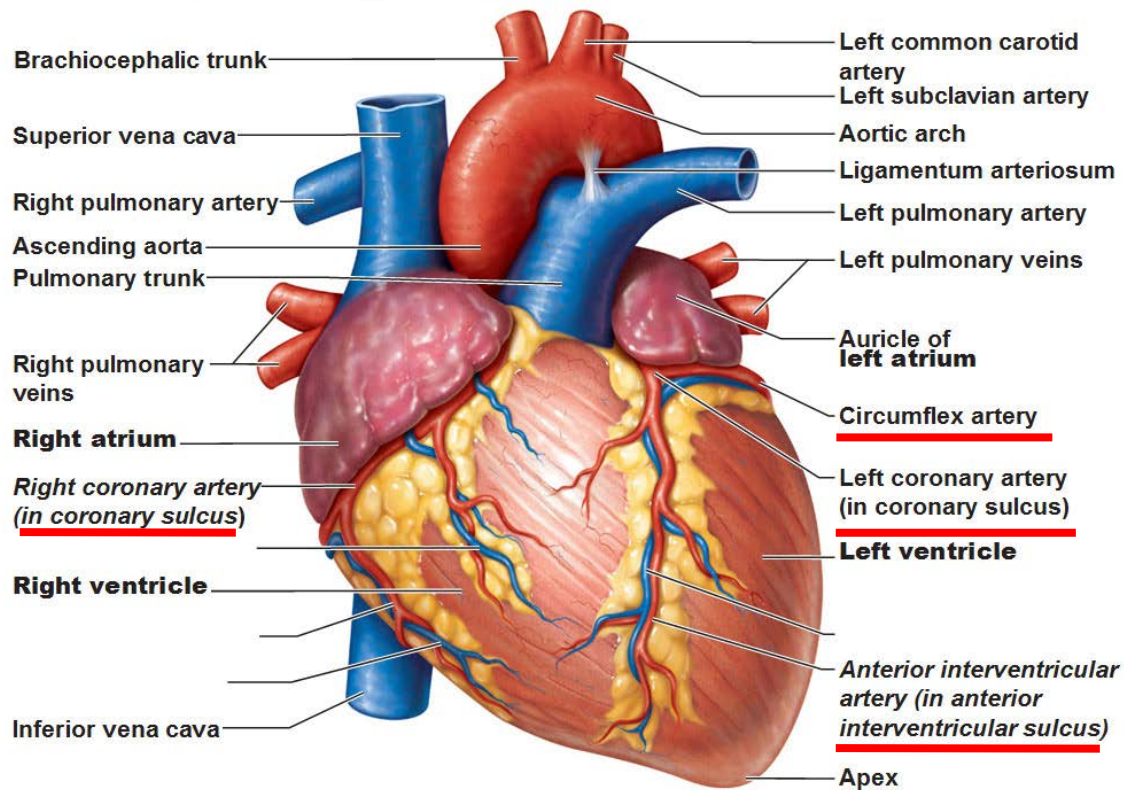


Figure 4. Gross anatomy of the human heart.

Gross anatomy of the human heart outlining major anatomical structures and vessels. The labels underlined in red indicate the locations of the coronary arteries. These are the vessels susceptible to CAD.

These cholesterol and lipid plaques lead to vascular ischemia (reduced blood flow) and a condition called atherosclerosis. Atherosclerosis results from a buildup of cholesterol and fat that together harden and narrow the diameter of coronary arteries (Fig. 5).

Coronary Artery Disease

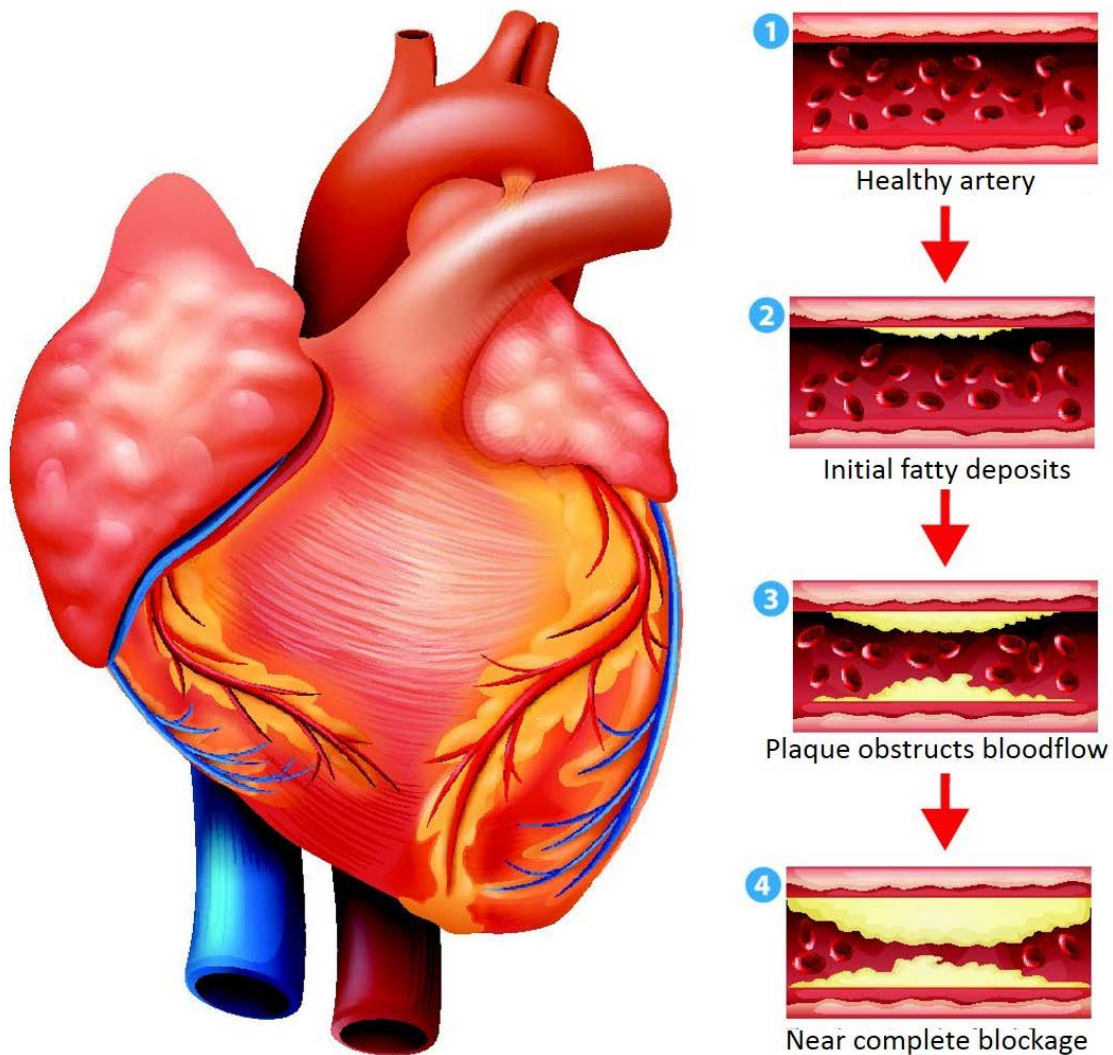
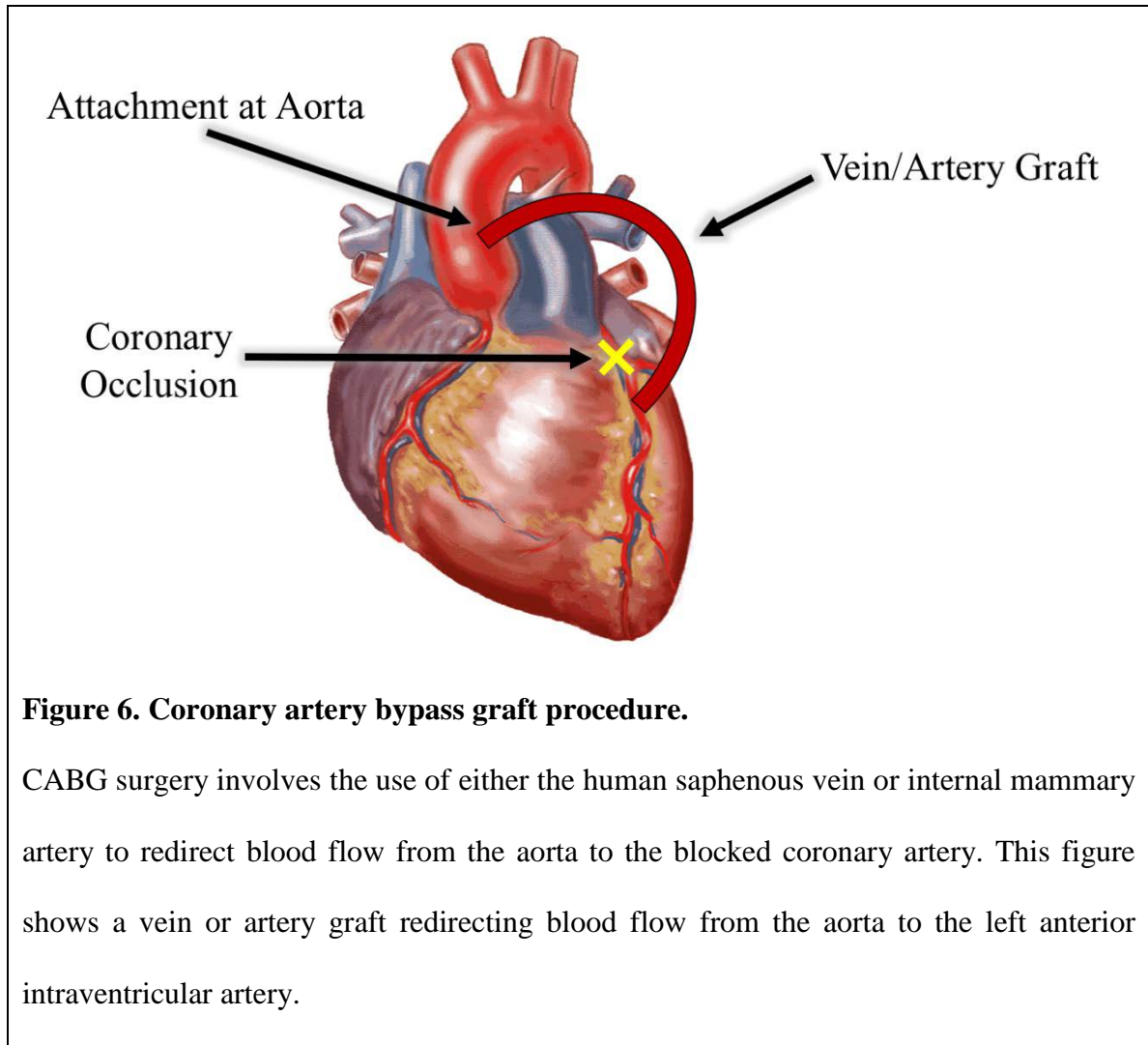


Figure 5. Vessel occlusion from atherosclerotic plaque buildup.

As fatty lipid plaques begin to accumulate in vessels, the diameters of these vessels begin to decrease as the disease progresses from Stage 1, pictured above, to the final Stage 4 at which there is near complete blockage of the artery.

This narrowing limits the flow of oxygen-rich blood to the myocardial tissue of the heart, and as a result this narrowing leads to the development of CAD (38, 39). Were these plaques to rupture inside the vessels, the rupture would promote an inflammatory response in which platelets, naturally found in the blood, would adhere to the site of damage on the endothelial wall of the vessel. If these platelets adhere and conglomerate so densely that they occlude the vessel, they can result in a blood clot or even a myocardial infarction (heart attack) (40). Considering in full the high mortality resulting from CAD, the risks associated with severe CAD, and the direct role of endothelial dysfunction in the development of CAD, it is important to understand how the endothelium functions in order possibly to prevent the onset and manifestation of CAD.

The most common interventional approach to correct vessel occlusion from the development of atherosclerosis and CAD is a surgical procedure known as coronary artery bypass graft (CABG) surgery. This procedure involves first the removal of either the patient's saphenous vein (HSV) from the leg or the internal mammary artery (IMA) and then the grafting of one of these vessels onto the heart to fulfill the role of an occluded coronary artery. The HSV graft is used primarily for blockages of the anterior descending coronary arteries, while the IMA graft is used for posterior coronary artery congestion (Fig. 4). CABG involves the bypassing of blood flow around the sections of a coronary artery congested with atherosclerotic plaques. The effect is that normal blood and oxygen flow is returned to the muscle of the heart (myocardium) (33) (Fig. 6).



While both vessel grafts are effective in bypassing blood flow around the congested coronary artery, the long-term effectiveness or patency of these two grafts are markedly different. It is understood that the HSV grafts have clinically worse outcomes over the long term in comparison to the IMA grafts. Within ten years following operation, HSV graft patency is 61%, while IMA graft patency is 85% (12). Additionally, HSV grafts have high rates of initial failure up to 25% within twelve to eighteen months following surgery (15, 27). With graft failure, a secondary procedure is required to bypass the vessels damaged by the failed HSV graft. The reasons for the failure of these

grafts is unknown; researchers hypothesize that differences in the venous and arterial environment may cause aberrant regulation of specific gene expression patterns that result in blood vessel dysfunction (29).

Considering that the endothelial cell layer is the first line of physical contact between the vasculature and the flow of blood, the endothelial cell layer provides an important role in maintaining overall vessel health and normal physiology (47). Disease pathophysiology of the vasculature, such as atherosclerosis and intimal hyperplasia (IH), stems directly from damage and disruption to this endothelial cell layer (43). Intimal hyperplasia is a disease caused by aberrant thickening of the sub-endothelial intimal layer of a blood vessel (32). Changes in vascular shear stress are suggested to be one of the early causes of downstream vessel dysfunction and IH following CABG procedures (23).

Given the significant role of the endothelium in the presentation of normal and abnormal physiology of the vasculature and overall cardiovascular system, it is important to understand the role of endothelial function and dysfunction in cardiovascular disease.

1.6 Fluid Shear Stress and Vascular Endothelium

Under normal physiological conditions, the vascular endothelium is constantly exposed to a variety of physical stressors called hemodynamic forces. The most commonly described hemodynamic forces are circumferential hoop stress and shear stress. Circumferential hoop stress is caused by the circumferential distension of a vessel wall by changes in blood pressure (28). Shear stress (expressed in units of dynes/cm²), the focus of this thesis, is the force per unit area created when a tangential force such as blood (or any fluid) flows parallel across a surface like the vascular endothelium.

Depending on the viscosity of the fluid, the length of chamber or vessel, the change in pressure across chamber or vessel, and the radius of the chamber or vessel, it is possible to calculate the shear stress applied to the wall of the chamber or vessel (55) (Equation 1).

$$\tau = 4\eta \cdot \frac{Q}{\pi r^3}$$

τ = Shear Stress

η = Blood Viscosity

$$Q = \text{Blood Flow} = \text{Poiseuille's Law} = \frac{\Delta P \cdot \pi \cdot r^4}{8 \cdot \eta \cdot l}$$

ΔP = Pressure Difference Through a Blood Vessel

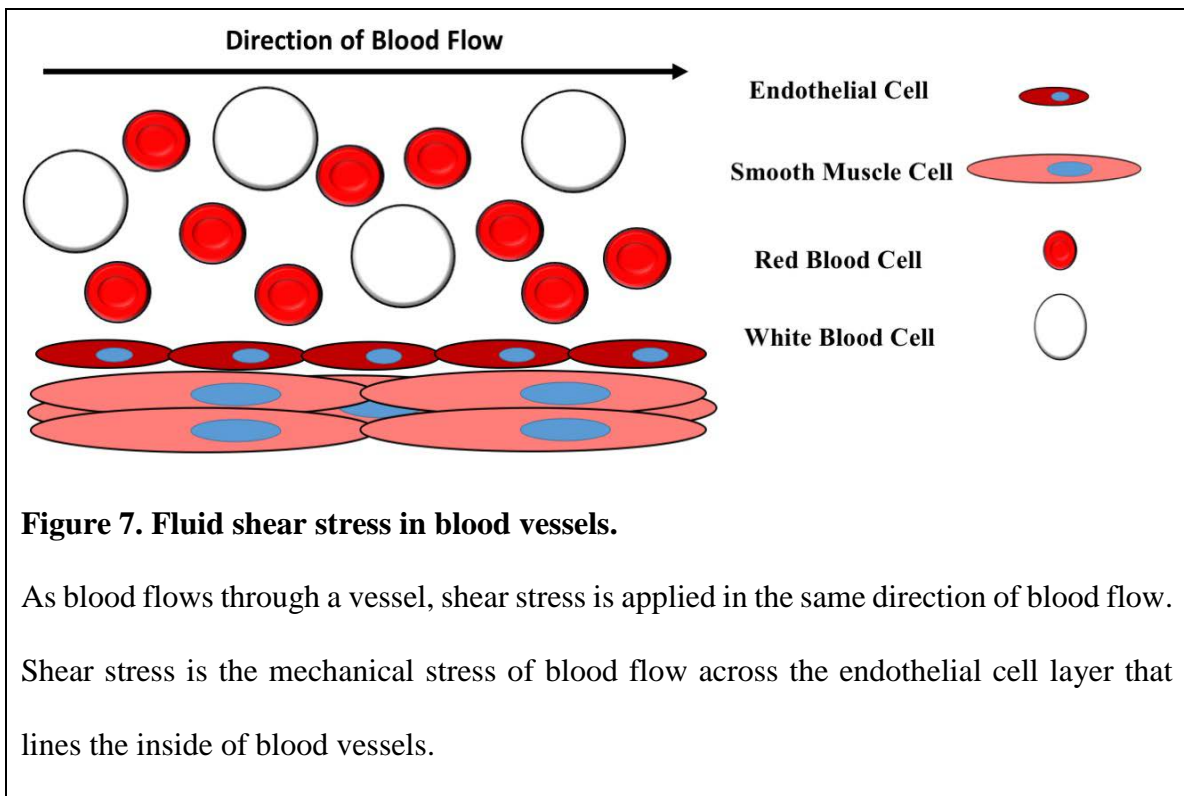
l = Length of Vessel

r = Vessel Radius

Equation 1. Equation for calculating shear stress in a closed vessel

Shear stress is exerted on vascular endothelium wherever there is blood flow (5) (Fig. 7). Shear stress on the wall of blood vessels and specifically on the vascular endothelium cannot be measured directly *in vivo*. However, shear stress can be calculated with the knowledge of blood's viscosity near the region of interest. While this might seem straightforward simply to measure the blood viscosity at the region of interest and use this information to calculate *in vivo* shear stress, this can be difficult due to a process called plasma skimming. During plasma skimming, the number of red blood cells interacting with and exerting a force upon wall of the vessel is inconsistent due to the relatively low level of red blood cells located directly adjacent to the vessel wall. In

addition, the diameter of a blood vessel varies significantly over the period of a single heartbeat. This provides additional difficulty in calculating blood viscosity and shear stress *in vivo*. It is estimated that shear stress ranges across the entire human vasculature from levels less than 10 dynes/cm² to levels upwards of 70 dynes/cm² (41).



1.7 Fluid Shear Stress Regulation of AQPs

As blood flows across the endothelial cell layer, the cell layer responds to this stress by regulating blood vessel physiology (47). The endothelial cell layer converts the mechanical stimuli of the shear stress into intracellular signaling pathways that can have effects on overall cellular function, proliferation, apoptosis, migration, permeability, remodeling, and gene expression (25).

While fluid shear stress in the context of this investigation is described as blood flow across endothelial cells, fluid shear stress exists anywhere there is fluid flowing

across a layer of cells such as in the epithelial layers of fluid filled structures. Such an environment exists in the proximal tubule of the kidney. As blood filtrate flows through the proximal tubule, fluid shear stress is exerted on the epithelial lining and results in modifications of downstream gene expression patterns. Of relevance is the regulation of AQP1 by fluid shear stress in these structures. It has been demonstrated by Pohl et al. that increases in fluid shear stress *in vitro* and *in vivo* upregulate the expression of AQP1 in the proximal tubular epithelial cells (42). These investigators demonstrated a significant increase in AQP1 expression under fluid flow, suggesting a shear stress dependent mechanism for regulating AQP1 expression.

In a separate study by Mun et al., this same regulation of AQP1 by shear stress was again demonstrated in vascular endothelial cells, suggesting a cell type-independent regulation of AQP1 by fluid shear stress. Increasing levels of shear stress from static conditions to flow conditions were shown to induce the expression of AQP1 in endothelial cells (34). It is hypothesized that this mechanically-driven induction of AQP1 under increasing shear stress serves as an early environmental sensor or biomarker as fluid shear stress increases from a venous to arterial environment.

1.8 Statins and AQPs

Statins belong to the class of drugs called HMG-CoA reductase inhibitors. These drugs function as cholesterol-lowering agents. They inhibit the rate-limiting step in the biochemical synthesis of cholesterol by direct inhibition of the enzyme HMG-CoA reductase (17). An essential sterol in the human body, cholesterol in normal concentrations serves in a wide variety of physiological roles including maintaining cell

membrane fluidity. However, increased amounts of low-density lipoprotein (LDL) cholesterol are detrimental to overall cardiovascular health. Increased plasma levels of LDL cholesterol can lead to a buildup of atherosclerotic plaques in blood vessels that directly affects the normal function of the vascular endothelium. These atherosclerotic plaques can cause damage to the overall vasculature by means of vascular inflammation, occlusion, and vessel wall thickening from IH (31). These effects of increased LDL cholesterol are the reasons why statins are commonly prescribed to those individuals suffering from CAD and are undergoing CABG procedures. This prescription is to prevent the accumulation of atherosclerotic plaques in newly implanted grafts as well as to prevent the regression of the vessel into significant coronary atherosclerosis (9).

Statins function as cardioprotective agents that reduce the risk of CAD and other cardiovascular diseases resulting from excessive LDL cholesterol. The current understanding of the cholesterol-independent cardioprotective effect of statins is that statins reduce endothelial permeability and vascular inflammation through the inhibition of Rho GTPases (3). These Rho GTPases function as molecular switches that regulate signal transduction and gene expression in endothelial cells (9). Through reducing endothelial permeability to immune cells such as leukocytes, statins reduce vascular inflammation that can lead to downstream vascular disease.

It has been observed that statins demonstrate a regulatory effect on certain members of the AQP family independent of their cholesterol-lowering effect. Statins regulate the expression of AQP1, -8, and -9 in cardiomyocytes following ischemic-reperfusion injury. Similar to the shear-stress induction of AQP1 in the proximal tubular epithelial cells and in HUVECs, AQP1, -8, and -9 are upregulated in the cardiomyocytes

after an ischemic event followed with reperfusion (24). This ischemia-reperfusion triggered induction of AQP1, -8, and -9 is prevented with the pretreatment of these cells with the hydrophilic statin, SimvastatinTM. The effect of SimvastatinTM was effectively counteracted by the addition of a protein-kinase A (PKA) inhibitor. These findings suggest that simvastatin exerts a cholesterol- and tissue-independent function in regulating AQPs through a PKA-dependent manner (24). This might additionally suggest a cholesterol-independent target of statin drugs in regulating AQPs and specifically AQP1 in tissue under flow conditions.

1.9 Hypothesis

The objective of this investigation is to investigate the role of the hydrophilic statin PravastatinTM on the regulation of AQP1 in vascular endothelial cells under static and venous fluid shear stress conditions.

It is likely that the cardioprotective function of statin drugs is a result of the effect that statins have in different cell types in regulating AQPs rather than exclusively on the lowering of LDL cholesterol (24, 34, 42). Previous findings have demonstrated that increases in shear stress from static to venous to arterial levels of shear stress can increase the expression of endothelial AQP1 (34). Given this understanding that AQP1 is upregulated in response to increasing levels of shear stress, and given that it is the increase in shear stress between a venous and an arterial environment that is proposed to be one of the reasons for CABG vein graft failure, it is possible that AQP1 might serve as a target for drug therapy focused on improving graft performance. In addition to the hypothesis that an increase in shear stress is a potential cause of CABG vein graft failure,

it is also hypothesized that increasing vascular permeability and inflammation can lead to graft failure. Aquaporin 1-null humans had significantly reduced vascular permeability in the vessels of the lungs as compared to normal humans (21). If it is proposed that reducing vascular permeability is a solution to preventing vein graft failure, then the absence or reduction of AQP1 in the vasculature would be optimal. A drug that could reduce this shear-stress dependent induction of AQP1 or even block its expression would function to mitigate the physiological stress that is caused by transplanting a vein graft to an arterial role in the heart.

Therefore, it is proposed that the reduction in vascular endothelial permeability caused by statin drugs functions through the regulation of AQP1 expression and subcellular localization in HUVECs rather than exclusively through a cholesterol-lowering mechanism. This suggests that statins might serve a role in the regulation of AQP1 in the vascular endothelium that supersedes the cholesterol-lowering purpose of statins and provides a cardioprotective benefit of reduced endothelial permeability.

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Chapter 2:

PravastatinTM Suppresses Venous Shear Stress Dependent Induction of Aquaporin 1 Protein Expression in Human Umbilical Vein Endothelial Cells *in vitro*.

2.1 Abstract

Background: Aquaporins (AQPs) are transmembrane water channels that facilitate osmotically-driven transmembrane water flux. AQP1 is abundantly expressed in vascular endothelium, where it functions in endothelial cell migration, wound healing, and cell volume regulation in response to mechanical stimuli. AQP1 protein abundance is enhanced in cultured human umbilical vein endothelial cells (HUVECs) in response to fluid shear stress. Statins, a pharmacological class of HMG-CoA reductase inhibitors, suppress the early development of vein graft intimal hyperplasia by improving endothelial cell function, most likely by affecting endothelial response to arterial shear stress. Statins may also differentially regulate the expression of specific AQPs by altering their abundance and/or subcellular localization. Therefore, it is hypothesized that AQP1 may function as a component of the mechanosensory complex in endothelial cells and therefore is subject to shear stress-induced gene regulation and modulation of expression by statins.

Methods: HUVECs were cultured in microfluidic chambers under static (0 dynes/cm²) and venous fluid shear stress (6 dynes/cm²) in the presence or absence of 5 μ M PravastatinTM. Fluorescent immunocytochemistry was used to detect AQP1 protein expression. Images were captured using confocal laser scanning microscopy. Mean

fluorescent intensity (in arbitrary units) was determined using ImageJ software and normalized to control conditions (time zero, static culture with no Pravastatin™).

Twenty-five to thirty cells from two independent experiments were analyzed for each experimental condition.

Results: AQP1 expression increased 1.4-fold ($p < 0.05$) after 24 hours, and 1.5-fold ($p < 0.05$) after 48 hours in cells cultured under venous fluid shear stress as compared to the control. This shear stress-induced expression was blocked by incubation with Pravastatin™ and reduced by 27% ($p < 0.05$; control vs. venous shear stress with Pravastatin™). Pravastatin™ had no effect on AQP1 expression in static cultures ($p > 0.05$ vs. control). These data indicate that Pravastatin™ suppresses shear stress dependent induction of AQP1 in cultured HUVECs.

Conclusions: Results show that the shear stress-dependent induction of AQP1 protein expression is suppressed by Pravastatin™. Results further demonstrate a potential function of statin drugs in regulating the expression of AQPs that is both independent of their cholesterol-lowering function and related to the regulation of AQP1 in vascular endothelial cells.

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2.2 Introduction

Aquaporins (AQPs) belong to the major intrinsic family of transmembrane proteins and function to facilitate osmotically-driven transmembrane water flux (1, 29). Of the 13 human AQPs, Aquaporin 1 (AQP1) is abundantly expressed in the vascular endothelium where it has been demonstrated to function in endothelial cell migration, wound healing, and cell volume regulation in response to mechanical stimuli (9, 12, 17). As blood flows across the vascular endothelium, the endothelial cell layer is exposed to a tangential mechanical force called laminar fluid shear stress (7, 8). In response to this mechanical stimulus, endothelial cells regulate whole blood vessel physiology (30). This response involves the conversion of mechanical stress into downstream intracellular signaling pathways that have an overall effect on regulating cellular function, proliferation, apoptosis, migration, permeability, and remodeling as well as endothelial cell gene expression (22).

Considering that the endothelium is the first line of physical contact between the vasculature and the flow of blood, the endothelial cell layer serves an important function in maintaining overall vessel health and normal physiology (30). Disease pathophysiology of the vasculature, such as intimal hyperplasia (IH), stems directly from damage and disruption to this endothelial cell layer (30). Given the significant role of the endothelium in normal and abnormal physiology of the vasculature and overall cardiovascular system, it is important to understand the role of endothelial cell function and dysfunction in cardiovascular disease.

It is known that fluid shear stress regulates the expression of members of the AQP family, specifically AQP1, *in vitro* and *in vivo*. Of the 13 isoforms of AQPs, AQP1 is

specifically expressed in endothelium located outside the brain such as in the microvessels of the kidney, lung and airways, secretory glands, skeletal muscle, pleura, and peritoneum (9–11, 13, 26). In the proximal tubule of the kidney, an increase in fluid shear stress upregulates the expression of AQP1 in proximal tubular epithelium and affects the trafficking of AQP1 to the apical membrane of these epithelial cells (15). This increase in expression and membrane localization induced by fluid shear stress suggests a shear stress dependent mechanism for regulating AQP1 expression and intracellular distribution. Similarly, *in vitro*, a change in the level of shear stress from static culture conditions to flow conditions induced the expression of AQP1 in endothelial cells, suggesting a cell-type independent regulation of AQP1 by changes in fluid shear stress (24).

Statin drugs belong to the class of pharmaceuticals called HMG-CoA reductase inhibitors that function as cholesterol-lowering drugs by inhibiting the rate-limiting step in the biochemical synthesis of cholesterol (18). The statin class of drugs has been proposed to have a cholesterol-lowering independent effect on the expression of varying AQPs. Statins are known to regulate protein expression of AQP1, -8, and -9 in cardiomyocytes following ischemic-reperfusion injury. AQP1, -8, and -9 are upregulated in cardiomyocytes after an ischemic event followed with reperfusion (21). This ischemia-reperfusion triggered induction of AQP1, -8, and -9 is prevented with the pretreatment of the cardiomyocytes with the hydrophilic statin, SimvastatinTM, resulting in inhibiting cardiomyocyte swelling due to water influx. This SimvastatinTM prevented induction of AQP1, -8, and -9 in cardiomyocytes was effectively counteracted by the addition of a protein-kinase A (PKA) inhibitor, suggesting that statins might exert a cell-type

independent effect in regulating AQP expression (21). Aquaporins might serve as targets of statins in various cell types under flow conditions.

The objective of this investigation is to determine the role of PravastatinTM, a hydrophilic statin drug, on the regulation of AQP1 protein expression in human umbilical vein endothelial cells (HUVECs) under static and venous fluid shear stress conditions *in vitro*. It is suspected that the function of statin drugs independent of their cholesterol-lowering primary utility is a result of the effect that statins might have in endothelial cells, specifically HUVECs, on the regulation of AQP1. It is therefore hypothesized that the fluid flow induction of AQP1 observed *in vitro* (24) will be altered by administering PravastatinTM in an *in vitro* model of fluid shear stress in HUVECs.

2.3 Materials and Methods

Cell Culture of Human Umbilical Vein Endothelial Cells (HUVECs)

Human umbilical vein endothelial cells harvested from normal full-term umbilical cord veins (Coriell Cell Repositories, Line WC00103) were cultured in complete cell culture media (CCCM, Lonza CC-3156) and supplemented with EBM-2 Bulletkit (Lonza CC-3162) and 7.5% Fetal Bovine Serum (GE Healthcare Life Sciences SH30071.03) in cell culture flasks coated with 1% gelatin. HUVECs were incubated in a CO₂ Incubator at 37°C and 5% CO₂ (Barnstead International Model 490).

Exposing HUVECs to venous fluid shear stress and PravastatinTM

Human umbilical vein endothelial cells were seeded at 5×10^5 cells/ml (static conditions) or at 2×10^6 cells/ml (flow conditions) in 1% gelatin-coated microfluidic

chambers (0.4 mm ibidi® μ -slides: ibidi 80601), and incubated at 37°C and 5% CO₂ in CCCM + Supplements for 24 hours prior to exposure to 5 μ M Pravastatin™ (EMD-Millipore 524403) and/or to the application of “venous” fluid shear stress (6 dynes/cm²) for 24 or 48 hours. Cells after the initial 24-hour incubation with no Pravastatin™ and no shear stress were used as a “Static 0 Hr” control for the purpose of data standardization.

Shear stress was applied using the 913 Mityflex peristaltic pump. Complete cell culture media + supplements with or without Pravastatin™ was placed in an Erlenmeyer flask, and the bioreactor apparatus was setup as described in Chambers (4) in a sterile Class II biosafety cabinet (Labconco Corporation 3620804). Temperature and CO₂ were monitored using GasLab® 2.0 software on an external computer setup. Cells were exposed to shear stress (6 dynes/cm²) for 24 and 48 hours. Complete cell culture media + supplements (with or without Pravastatin™) was replaced after 24 hours of culture under static and flow conditions.

Immunocytochemistry

Human umbilical vein endothelial cells seeded in microfluidic chambers were washed with 1X Phosphate Buffered Saline (PBS) (pH 7.4) and then fixed with 4% paraformaldehyde in PBS. Slides were blocked using 10% blocking serum [0.24 g Bovine Serum Albumin (Sigma Aldrich A9418), 600 μ L goat serum (Vector Laboratories S-1000), 30 μ L Tween-20 (Sigma P9416), and 5.3 mL of 1x PBS]. HUVECs were incubated at 4° C with 1:100 dilutions of primary antibody for AQP1 (Santa Cruz Biotechnology 32737), PECAM-1 (Santa Cruz Biotechnology 8306), or BMPER (Santa Cruz Biotechnology 377502).

Following primary antibody incubation, HUVECs were exposed to fluorescein-conjugated secondary antibodies at a 1:200 dilution in 1% blocking serum [(Goat Anti-Mouse IgM Antibody; Vector Laboratories FI-2020) (Goat Anti-Rabbit IgG Antibody; Vector Laboratories FI-1000)]. HUVECs were then exposed to a 1:100 dilution of Texas-Red Phalloiden (Life Technologies T7471) in 1% blocking serum to stain for F-actin, a cytoskeletal protein in HUVECs. Cell nuclei were stained using TOPRO-3 (Life Technologies T3605), a nuclear stain, diluted 1:500 in Vectashield fluorescent mounting media (Vector Laboratories H-1000). Slides were stored in the dark at 4°C. Immunofluorescence was detected using an Olympus Fluoview 1000 Laser Scanning Confocal Microscope, and images were taken of the HUVECs at 20x and 40x magnification.

Statistical Analysis

Immunoreactivity of AQP1, BMPER, and PECAM mean fluorescent intensity was quantified from twenty to thirty cells from each 20x magnification image using the “Freehand Selection Tool” provided in ImageJ, and averages and standard error of the means were calculated for graphical representation. A four-square grid was superimposed on the images and cells were sampled from the top left and bottom right quadrants of the images. Mean fluorescent intensity values were normalized to the Static 0 Hr and compared between experiments. All experiments were repeated and relative normalized values across replicates were compared for statistical replication. These mean fluorescent intensity values +/- standard error of the mean were represented as arbitrary units (a.u.). A blocking variable for experimental replicates was used to determine nonsignificant

differences between experimental replicates ($p>0.05$). Conditions were compared using an ANOVA with Tukey's Honest Significant Difference post-hoc correction.

Significance was determined at $p<0.05$.

2.4 Results

Effect of Venous Fluid Shear Stress on HUVECs

Initial experiments were focused on understanding how a change in fluid shear stress from static conditions to venous conditions might affect protein expression in HUVECs *in vitro*. Specifically, the experiments were designed to examine how a change in shear stress from a static environment to venous flow environment might affect the expression of AQP1.

Venous fluid shear stress induced AQP1 protein expression 1.4-fold relative to Static 0 Hour Control (Fig. 8, Panel F vs. Panel B), Static 24 Hours (Fig. 8, Panels E-H) and Venous Flow 24 Hours (Fig. 8, Panels I-L; Fig. 9, A; Fig. 9, B; $p<0.05$). AQP1 expression was induced 1.5-Fold after 48 hours of venous flow relative to Static 0 Hour Control (Fig. 8, Panel R vs. Panel B; Fig. 9, D; $p<0.05$). No significant difference in AQP1 expression was observed between Venous Flow for 24 hours as compared to cells cultured under Venous Flow for 48 hours (Fig. 8, Panel J vs. Panel R; $p>0.05$).

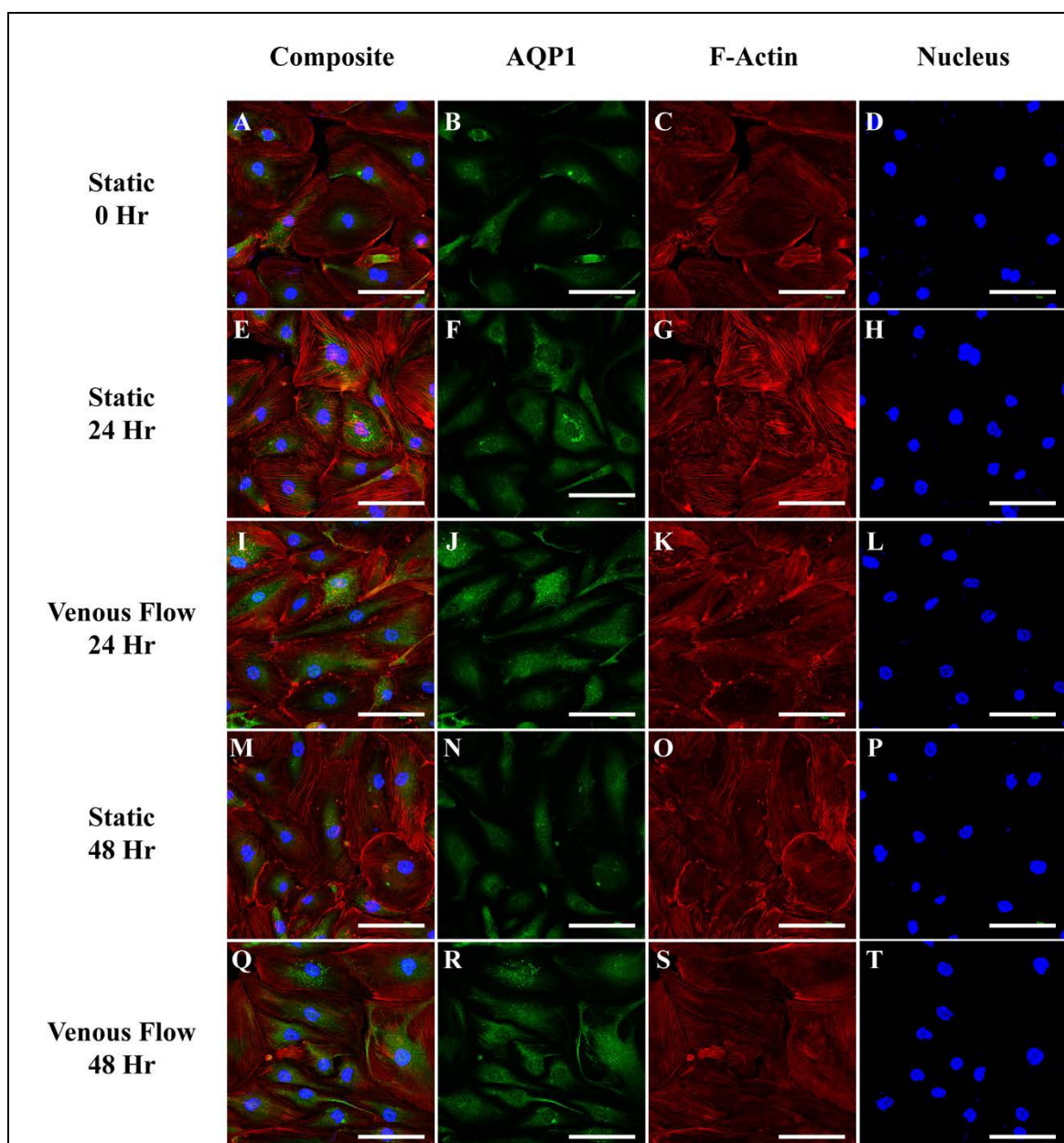
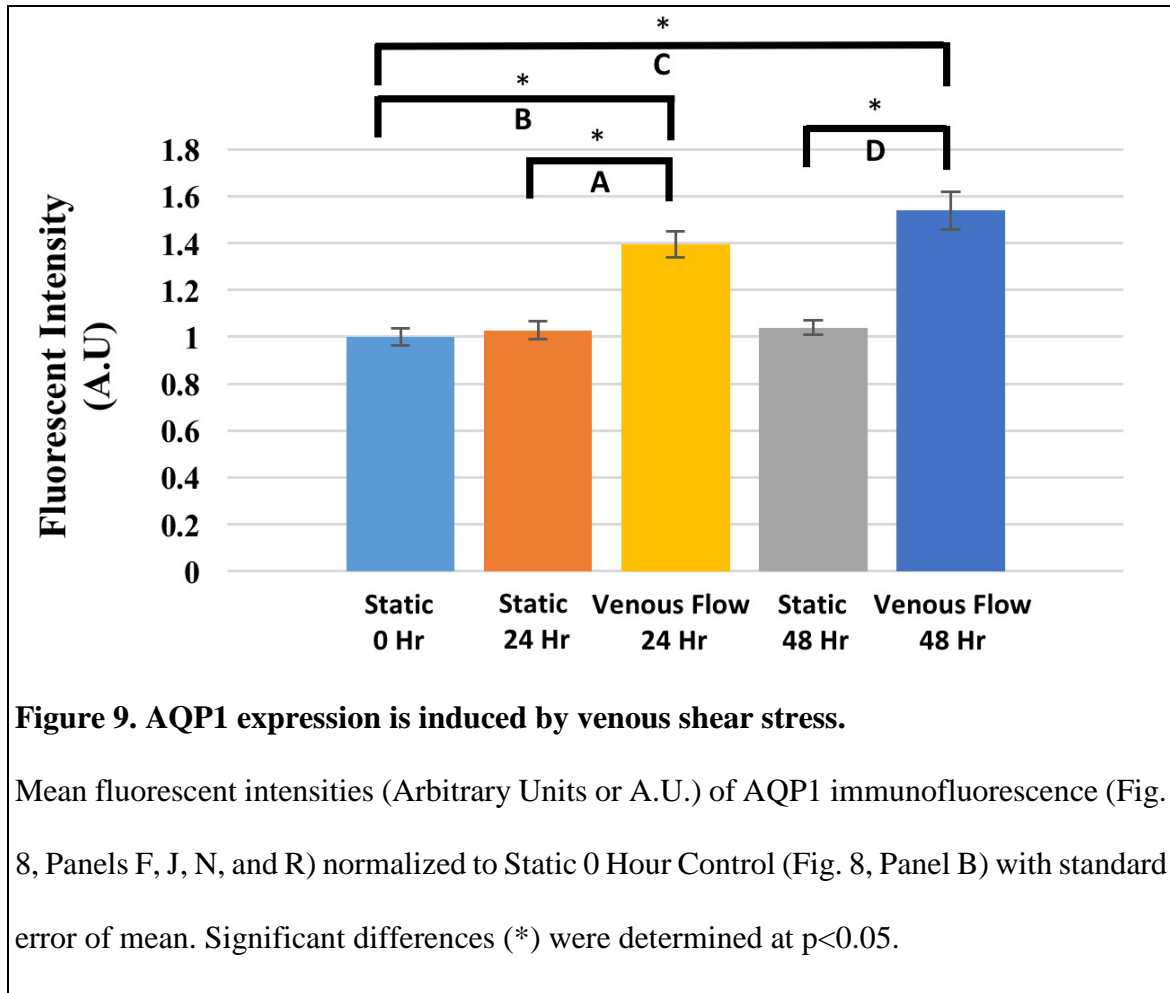


Figure 8. Venous fluid shear stress induces AQP1 expression in HUVECs.

Immunocytochemistry images of AQP1 (Green), F-Actin (Red), and the cell nuclei (Blue) acquired at 20x magnification of HUVECs cultured under static (A-H, M-P) and venous fluid shear stress conditions (I-L, Q-T) for 0 (A-D), 24 (E-L), and 48 hours (M-T). The direction of applied flow is from right to left. Scale Bar: 100 μ m.



Platelet Endothelial Cell Adhesion Molecule 1 (PECAM-1) was used as a positive marker for identifying non-differentiated endothelial cells (25). Relative to Static 0 Hour Control, the PECAM-1 expression did not change under static or venous flow conditions at 24 and 48 hours of culture indicating that shear stress has no effect on PECAM-1 expression, and indicates that the HUVECs maintained endothelial cell identity (Fig. 10, Panels B, F, J, N, and R; Fig 11; $p > 0.05$).

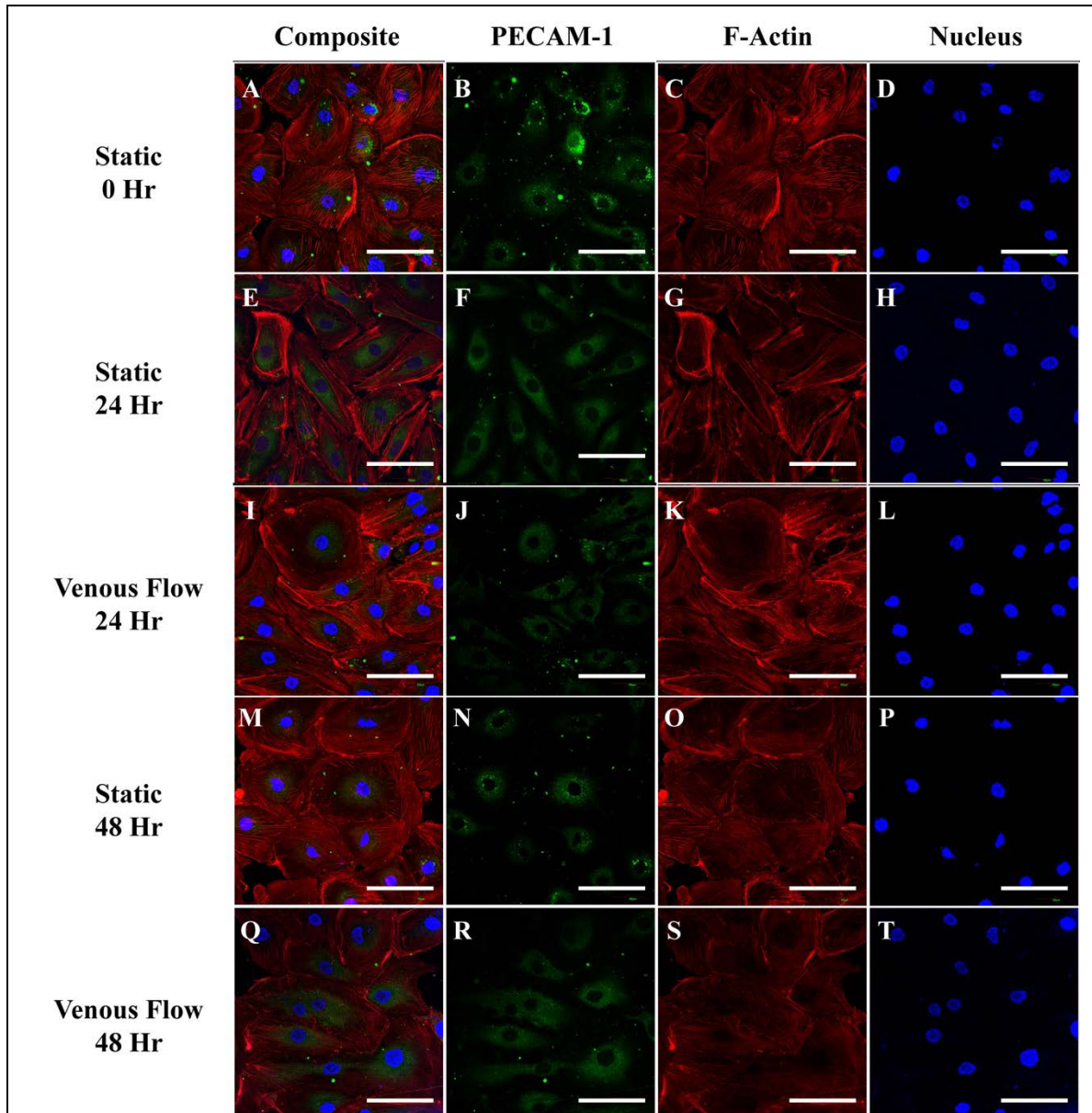


Figure 10. PECAM-1 expression is not affected by shear stress.

Immunocytochemistry images of PECAM-1 (Green), F-Actin (Red), and the cell nuclei (Blue) acquired at 20x magnification of HUVECs cultured under static (A-H, M-P) and venous fluid shear stress conditions (I-L, Q-T) for 0 (A-D), 24 (E-L), and 48 hours (M-T). The direction of applied flow is from right to left. Scale Bar: 100 μ m.

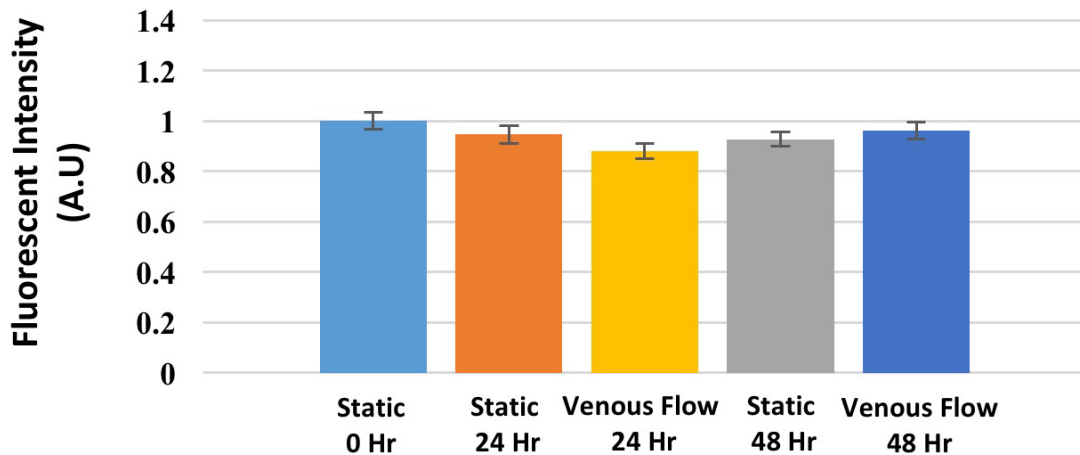


Figure 11. PECAM-1 expression is not affected by venous shear stress.

Mean fluorescent intensities (A.U.) (Fig. 10, Panels F, J, N, and R) normalized to Static 0 Hour Control (Fig. 10, Panel B) with standard error of mean. No significant differences across conditions were observed ($p > 0.05$).

Bone Morphogenetic Protein-Binding Endothelial Cell Precursor-Derived Regulator (BMPER) a protein that has been shown to be up-regulated in endothelial cells by PravastatinTM, was used as a positive control for the effect of PravastatinTM in HUVECs (16). Relative to Static 0 Hour Control, the BMPER expression remained constant under static or venous flow conditions at 24 and 48 hours of culture (Fig. 12, Panels B, F, J, N, and R; $p > 0.05$).

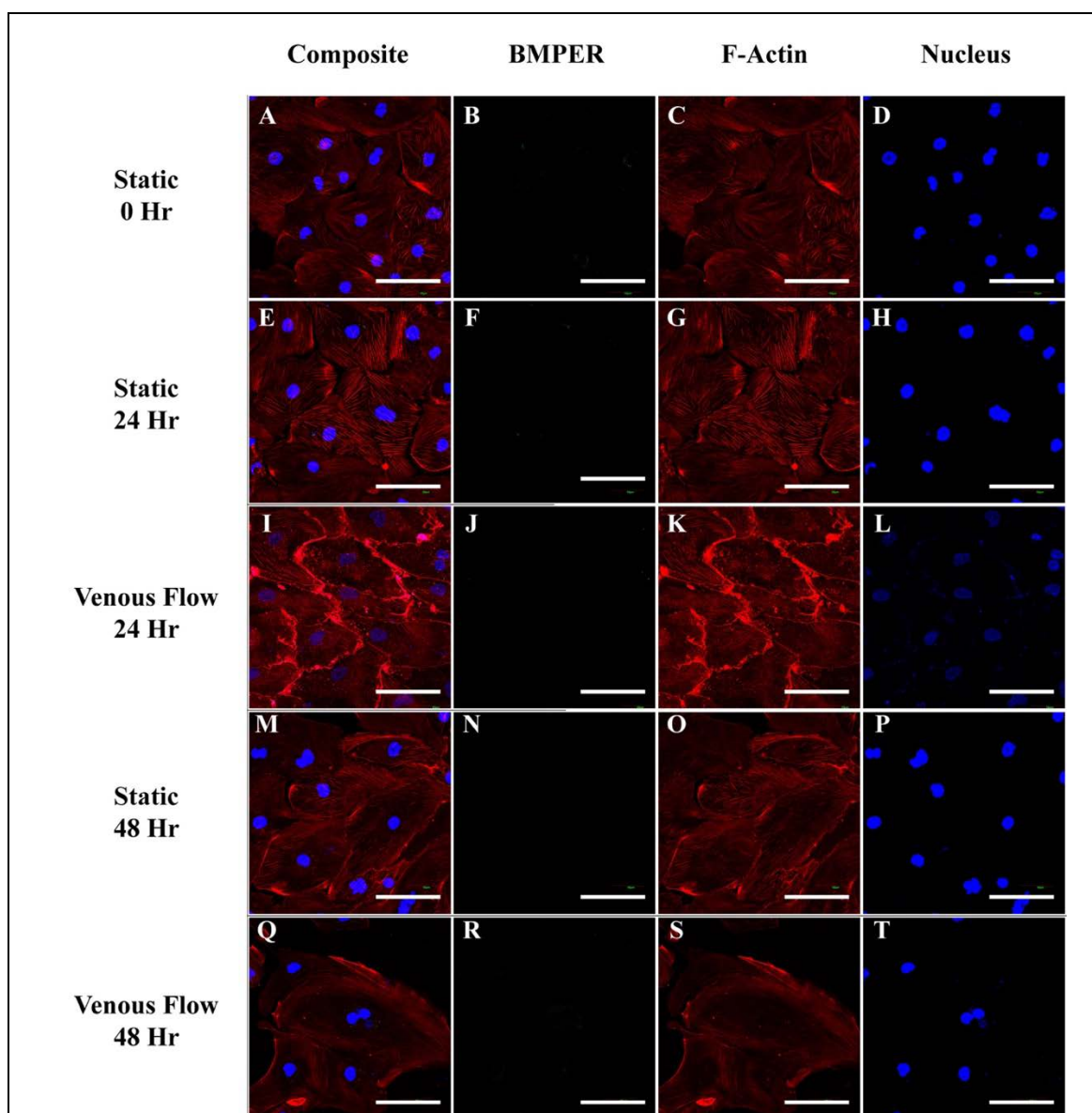


Figure 12. BMPER is not expressed in HUVECs cultured under static or venous shear stress conditions.

Immunocytochemistry images of Pravastatin™ positive control BMPER (Green), F-Actin (Red), and the cell nuclei (Blue) acquired at 20x magnification of HUVECs cultured under static (A-H, M-P) and venous fluid shear stress conditions (I-L, Q-T) for 0 (A-D), 24 (E-L), and 48 hours (M-T). The direction of applied flow is from right to left. Scale Bar: 100 μm .

Effect of 5 μ M PravastatinTM on Static Cultured HUVECs

PravastatinTM in CCCM was added to static HUVEC cultures to assess the effect of PravastatinTM on HUVECs independent of venous fluid shear stress. These experiments were performed to assess the effect of PravastatinTM on AQP1 expression in HUVECs cultured under static conditions. PravastatinTM has no effect on AQP1 protein expression in HUVECs cultured under static culture conditions after 24 and 48 hours of culture (Fig. 13, Panels B, F, J, N, R; Fig. 14; $p>0.05$).

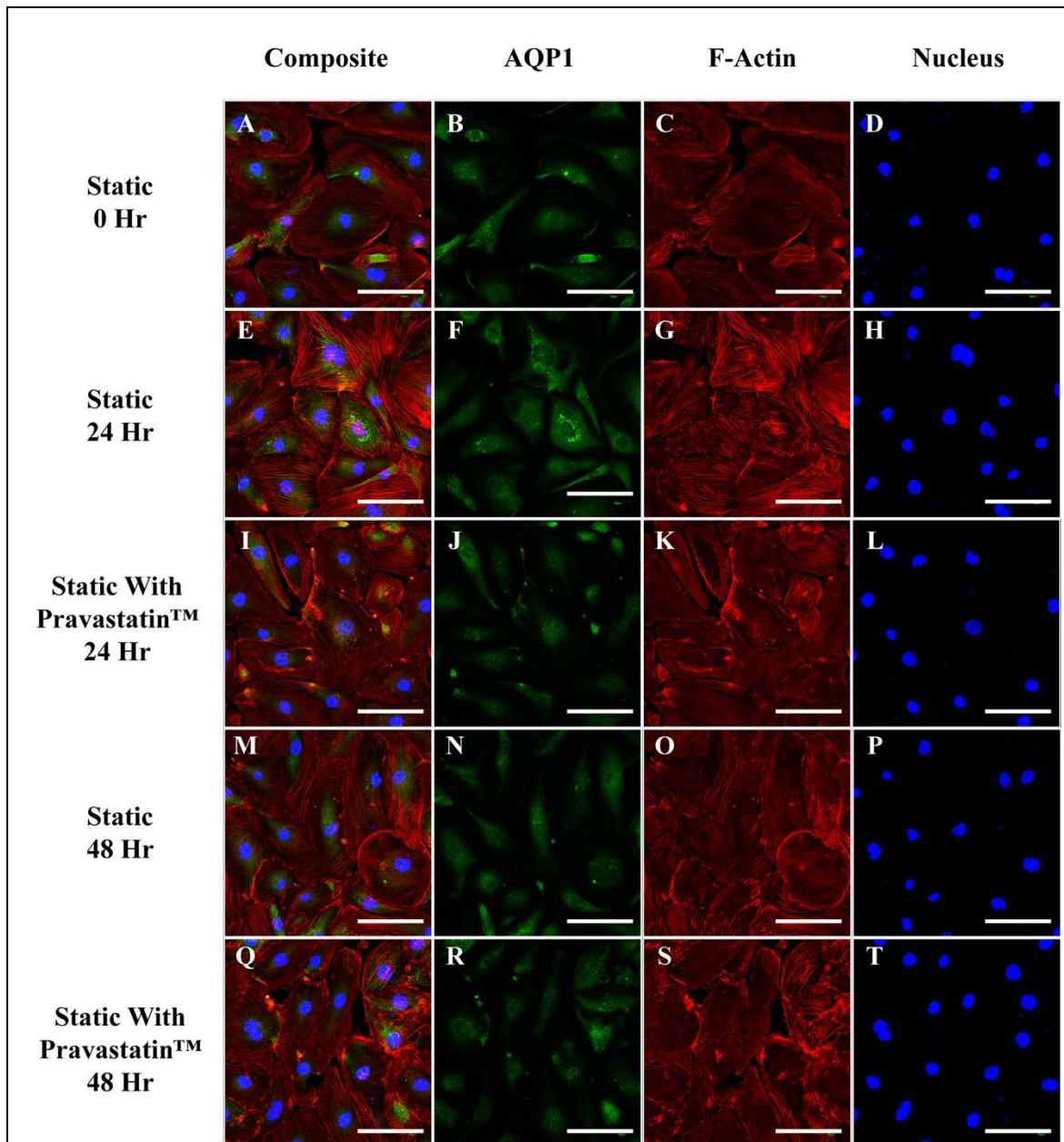
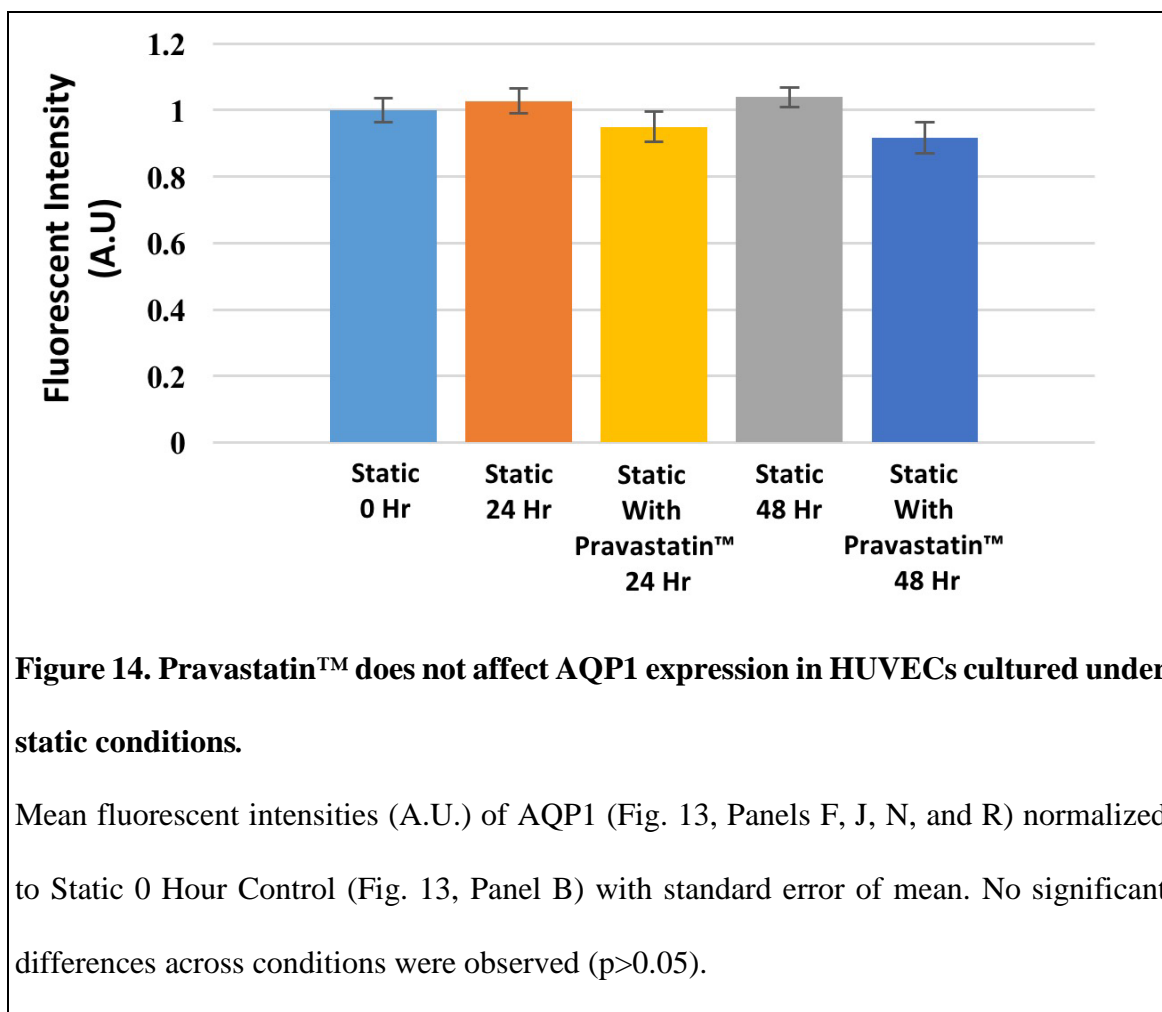


Figure 13. Pravastatin does not affect AQP1 expression in HUVECs cultured under static conditions.

Immunocytochemistry images of AQP1 (Green), F-Actin (Red), and the cell nuclei (Blue) acquired at 20x magnification of HUVECs cultured under static conditions (A-T) without Pravastatin™ (A-H, M-P) and with Pravastatin™ (I-L, Q-T) for 0 (A-D), 24 (E-L), and 48 hours (M-T). Scale Bar: 100 μ m.



Pravastatin™ did not affect PECAM-1 expression in HUVECs cultured under static conditions for 24 and 48 hours (Fig 15. Panels B, F, J, N, R; Fig. 16; $p>0.05$).

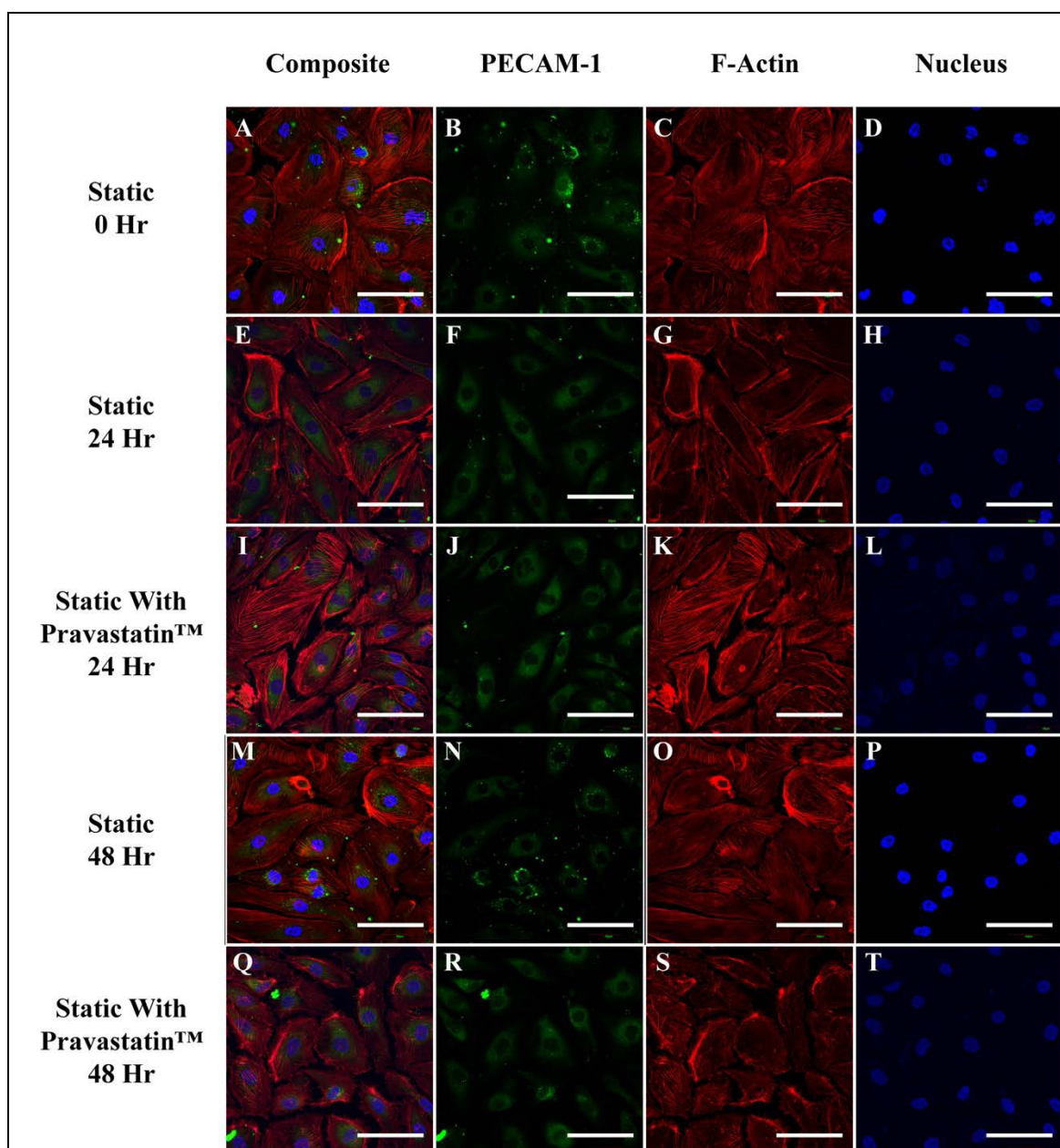
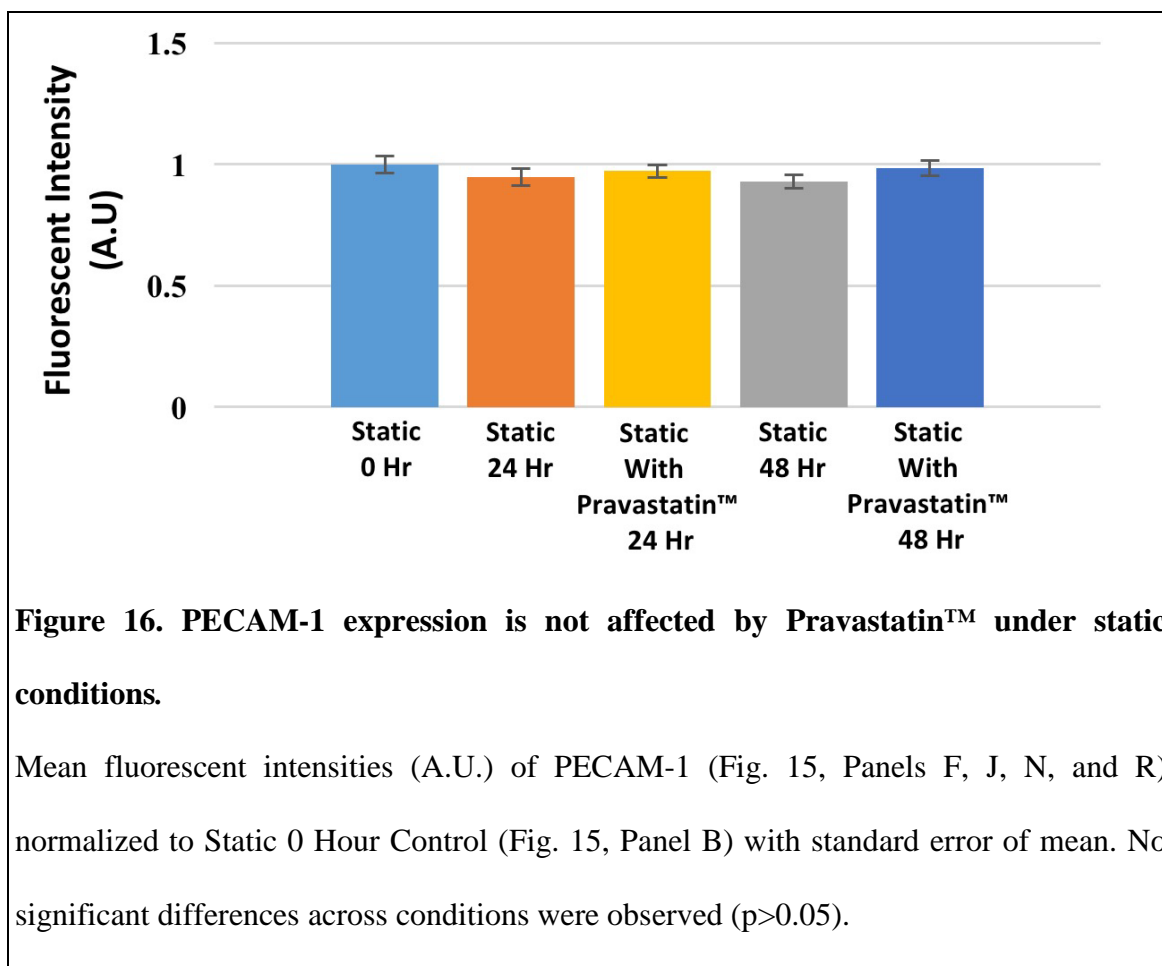


Figure 15. PECAM-1 expression remains constant in HUVECs cultured under static conditions with Pravastatin™.

Immunocytochemistry images of PECAM-1 (Green), F-Actin (Red), and the cell nuclei (Blue) acquired at 20x magnification of HUVECs cultured under static conditions (A-T) without Pravastatin™ (A-H, M-P) and with Pravastatin™ (I-L, Q-T) for 0 (A-D), 24 (E-L), and 48 hours (M-T). Scale Bar: 100 μ m.



After 24 hours (Fig. 17, Panels I-L) and 48 hours (Fig. 17, Panels Q-T) of static culture with 5 μ M Pravastatin™, BMPER expression is significantly increased relative to Static 0 Hour Control (Fig. 17, Panel J and R vs. Panel B; $p<0.05$).

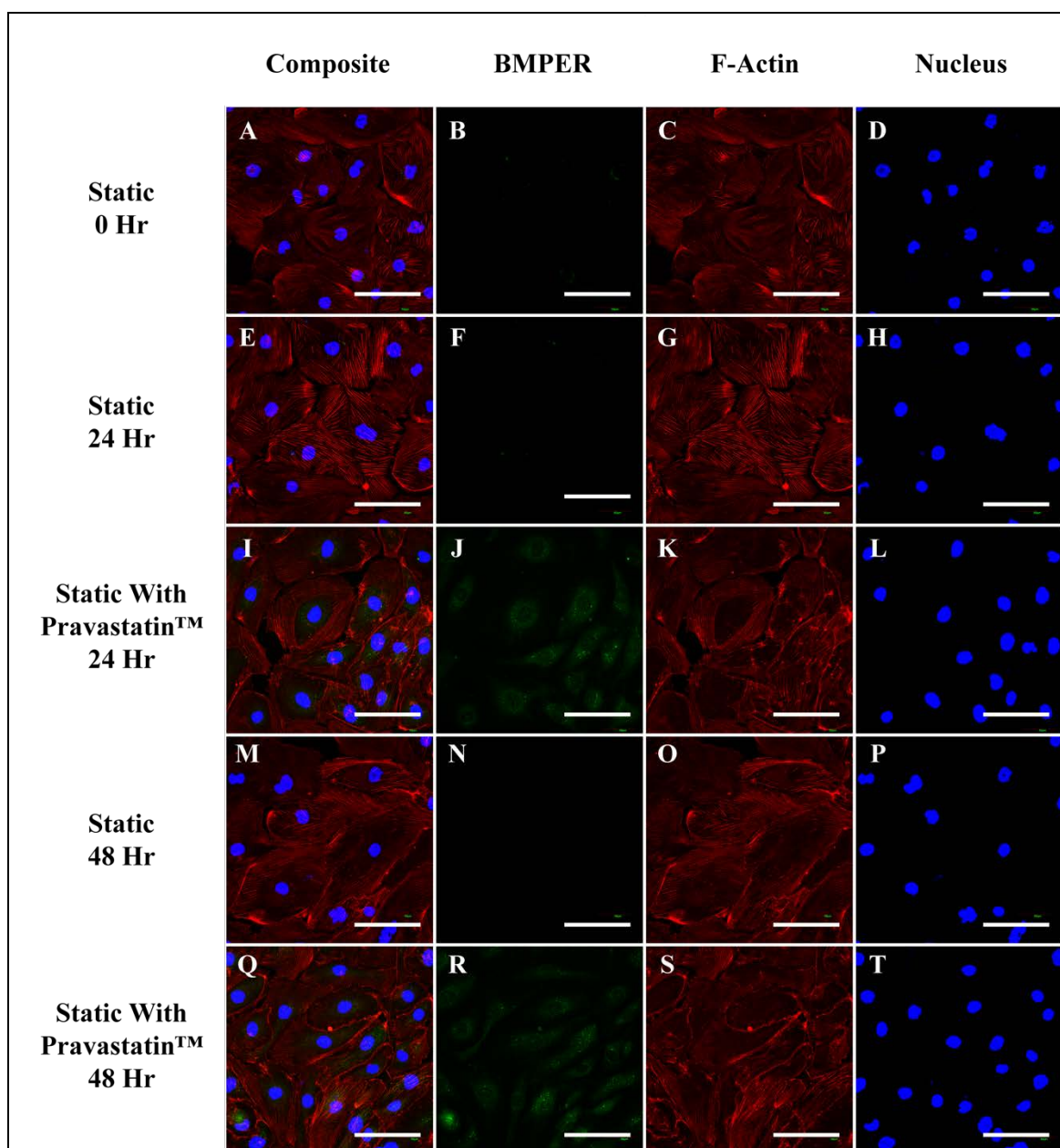


Figure 17. BMPER expression is increased in HUVECs cultured under static conditions with Pravastatin™.

Immunocytochemistry images of BMPER (Green), F-Actin (Red), and the cell nuclei (Blue) acquired at 20x magnification of HUVECs cultured under static conditions (A-T) without Pravastatin™ (A-H, M-P) and with Pravastatin™ (I-L, Q-T) for 0 (A-D), 24 (E-L), and 48 hours (M-T). Scale Bar: 100 μ m.

Effect of Venous Shear Stress and 5 μ M PravastatinTM on HUVECs.

PravastatinTM was added to HUVEC cultures under venous flow conditions to assess the interaction of venous shear stress and PravastatinTM on HUVECs. These experiments were performed to understand combinatorial effects of venous shear stress and PravastatinTM on AQP1 expression.

With the administration of 5 μ M PravastatinTM and the application of venous flow, the previously mentioned venous shear-dependent increase in AQP1 expression was repressed relative to Static 0 Hour Control (Fig. 18, Panels A-D; Fig. 19, A and E). After 24 hours of venous fluid shear stress with PravastatinTM (Fig. 18, Panels I-L), no venous shear-dependent increase in AQP1 expression was measured. AQP1 expression after 24 hours of venous flow and exposure to PravastatinTM (Fig. 18, Panel J) was reduced by 27% relative to Static 0 Hour Control (Fig. 18, Panel B; Fig. 19, B and D; $p < 0.05$). AQP1 expression after 48 hours of venous flow and exposure to PravastatinTM (Fig. 18, Panel R) was also reduced by 27% relative to Static 0 Hour Control (Fig. 18, Panel B; Fig. 19, C and F; $p < 0.05$). There was no significant difference measured between AQP1 expression after 24 hours and 48 hours of venous flow with PravastatinTM (Fig. 18, Panels J and R).

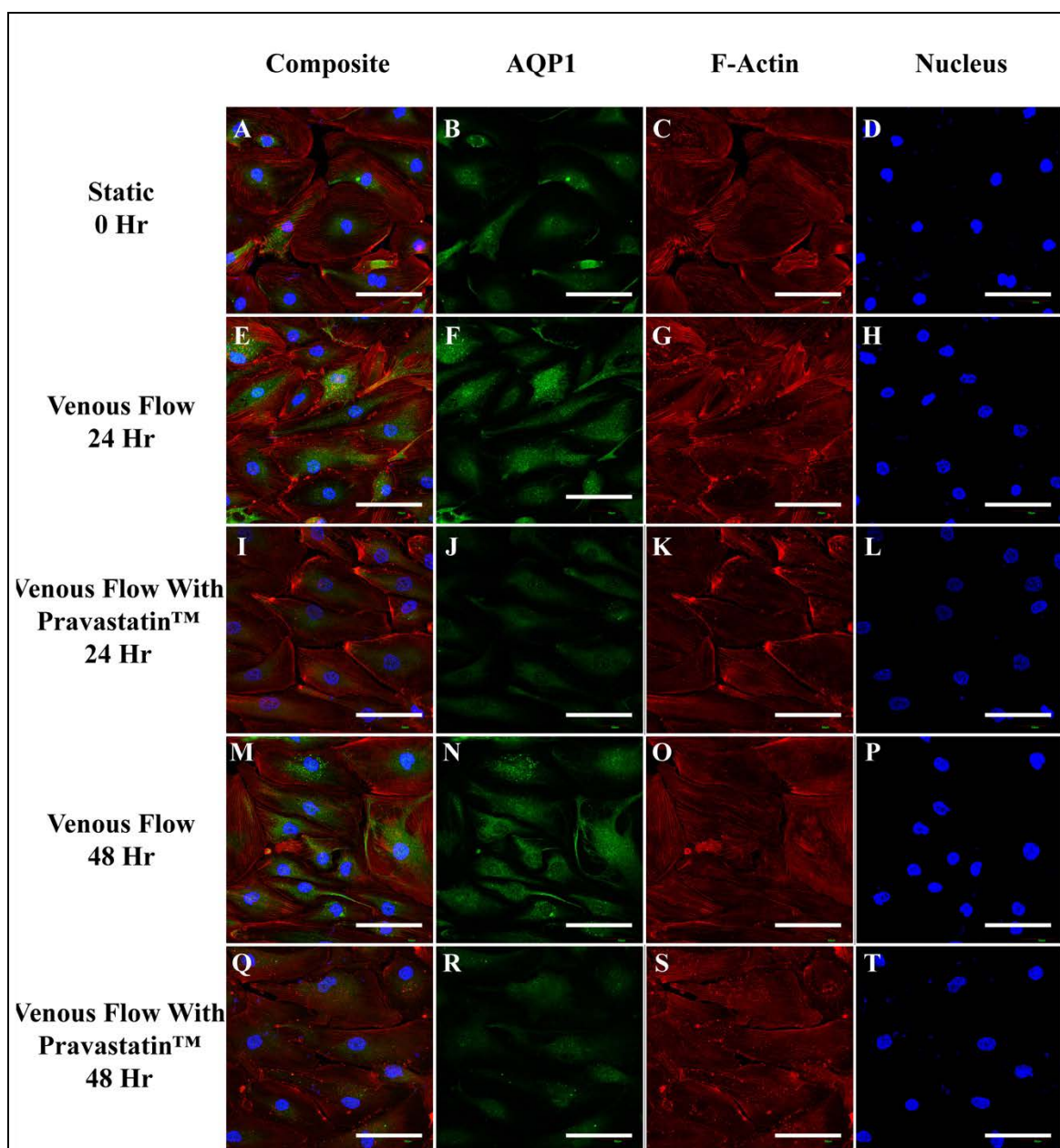
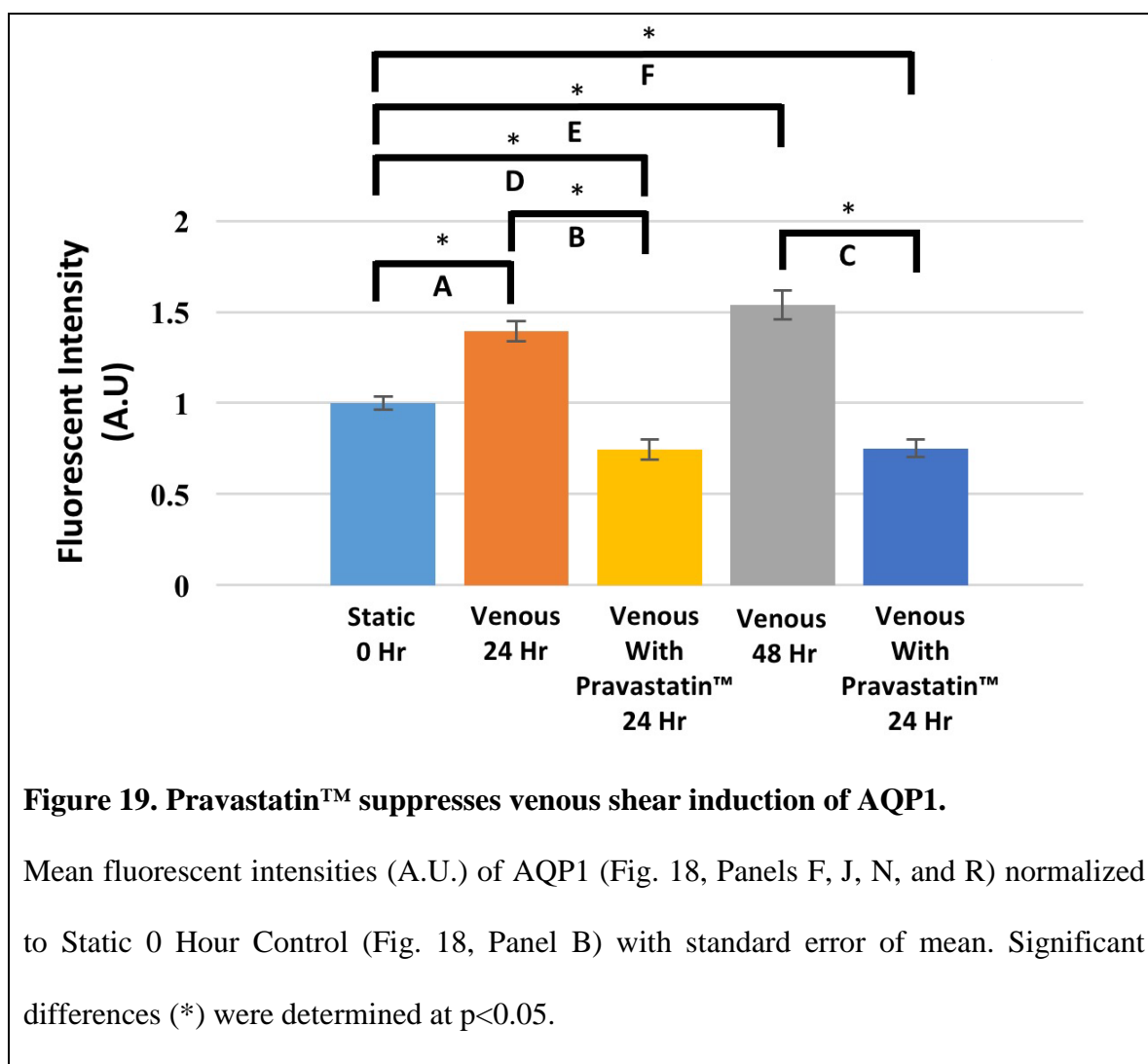


Figure 18. Venous shear induction of AQP1 is suppressed by Pravastatin™.

Immunocytochemistry images of AQP1 (Green), F-Actin (Red), and the cell nuclei (Blue) acquired at 20x magnification of HUVECs cultured under venous flow conditions (A-T) without Pravastatin™ (A-H, M-P) and with Pravastatin™ (I-L, Q-T) for 0 (A-D), 24 (E-L), and 48 hours (M-T). Venous flow was applied from left to right. Scale Bar: 100 μ m.



PECAM-1 expression remained constant in venous flow cultures exposed to 5 μ M Pravastatin™ relative to Static 0 Hour Control conditions. After 24 hours or 48 hours of venous flow culture with Pravastatin™ there was no significant alteration of PECAM-1 expression relative to Static 0 Hour Control conditions (Fig. 20, Panels J and R vs. Panel B; $p > 0.05$). PECAM-1 expression did not change under static or venous flow conditions at 24 and 48 hours of culture with and without Pravastatin™, indicating that shear stress and Pravastatin™ combined do not differentially regulate PECAM-1 expression or cause endothelial cell differentiation ($p > 0.05$).

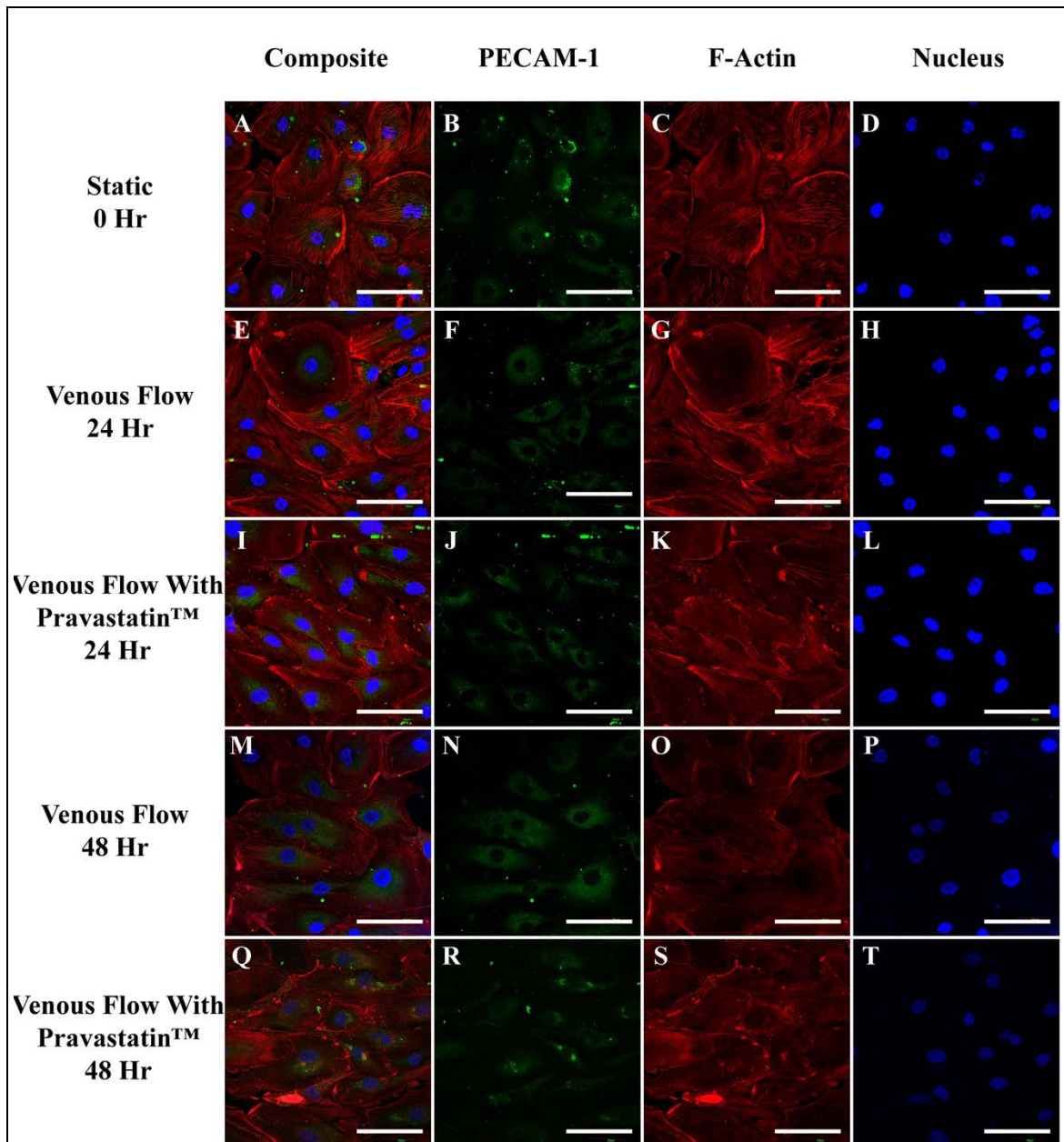


Figure 20. PECAM-1 expression is not affected by shear stress or Pravastatin™.

Immunocytochemistry images of PECAM-1 (Green), F-Actin (Red), and the cell nuclei (Blue) acquired at 20x magnification of HUVECs cultured under static (A-D) and venous flow conditions (E-T) without Pravastatin™ (A-H, M-P) and with Pravastatin™ (I-L, Q-T) for 0 (A-D), 24 (E-L), and 48 hours (M-T). Scale Bar: 100 μ m.

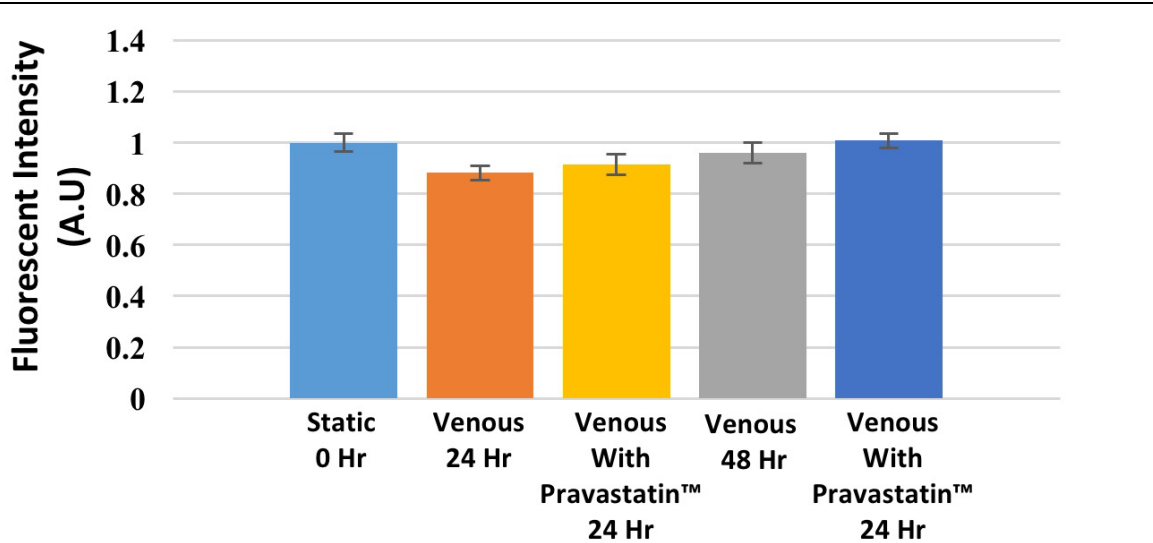


Figure 21. Shear stress and Pravastatin™ do not affect PECAM-1 expression.

Mean fluorescent intensities (A.U.) (Fig. 20, Panels F, J, N, and R) were normalized to Static 0 Hour Control (Fig. 20, Panel B) with standard error of mean. No significant differences across conditions were observed ($p>0.05$).

After 24 hours (Fig. 22, Panels I-L) and 48 hours (Fig. 22, Panels Q-T) of venous culture with 5 μ M Pravastatin™, BMPER expression is significantly increased relative to Static 0 Hour Control (Fig. 22, Panel J and R vs. Panel B; $p<0.05$). There is no significant difference when comparing BMPER expression after 24 and 48 hours under venous flow with Pravastatin™ (Fig. 22, Panels J and R) or between venous flow and static culture BMPER expression after 24 and 48 hours with Pravastatin™ (Fig. 17, Panels J and R; $p>0.05$). BMPER expression is only detectable with conditions using Pravastatin™.

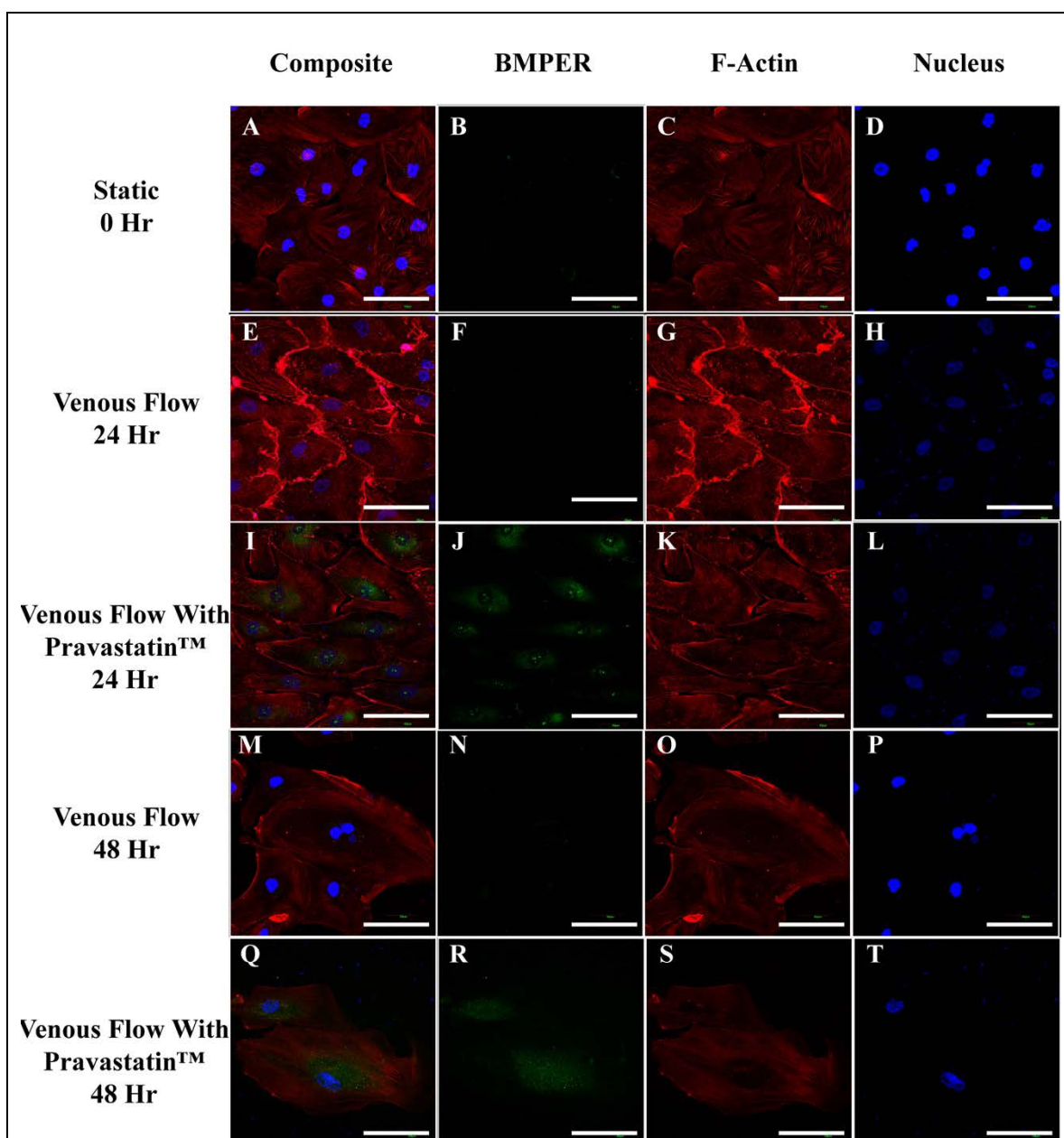


Figure 22. Pravastatin™ induces BMPER expression independent of shear stress.

Immunocytochemistry images of BMPER (Green), F-Actin (Red), and the cell nuclei (Blue) acquired at 20x magnification of HUVECs cultured under static conditions (A-T) and venous flow conditions (E-T) without Pravastatin™ (A-H, M-P) and with Pravastatin™ (I-L, Q-T) for 0 (A-D), 24 (E-L), and 48 hours (M-T). Scale Bar: 100 μ m.

2.5 Discussion

Fluid shear-stress dependent induction of AQP1 in vascular endothelial cells was confirmed *in vitro* as expected from previous investigations regarding the fluid shear-stress dependent regulation of AQPs, specifically AQP1 (24, 28). This might suggest a universal effect of shear stress on regulating AQP expression in multiple different cell types.

Specifically, in HUVECs this increase in overall abundance of AQP1 might serve to facilitate increasing endothelial cell volume under shear stress conditions. As the endothelial cells are exposed to an increase in fluid shear stress, the endothelial cells begin to align parallel to the direction of flow and elongate in response to flow. Cells elongating under flow conditions may result in cytosolic volume changes of the cell that in turn cause a compensatory response of increased AQP1 expression to compensate for an increased cytosolic volume (8). The increase in AQP1 under increasing shear stress suggests a potential mechanosensory signaling network for regulating AQP1 protein expression in HUVECs under shear stress.

Since the vascular endothelium and endothelial cells are constantly under fluid shear-stress *in vivo*, the most physiologically relevant understanding of the role of Pravastatin™ on AQP1 expression in HUVECs would have to be obtained using an *in vitro* recapitulation of venous fluid shear stress. Under static conditions with Pravastatin™, AQP1 expression is not affected ($p > 0.05$). However, with Pravastatin™ and the application of venous fluid shear stress, Pravastatin™ appears to not only block or prevent the shear-stress mediated induction of AQP1 in HUVECs but also further reduces levels of AQP1 expression to levels 27% less than those of control static

conditions ($p < 0.05$). This combinatorial regulation demonstrated by PravastatinTM and shear stress suggests a novel relationship between PravastatinTM, shear stress, and the regulation of AQP1.

While statins primarily function to reduce levels of low-density lipoprotein cholesterol, statins are suggested to have a cholesterol-independent cardioprotective effect by reducing endothelial permeability and vascular inflammation through the inhibition of Rho GTPases (3). Additionally, statins have been shown to regulate AQP1 protein expression by increasing the expression of Kruppel-like factor 2 (KLF2) (27). KLF2 is the transcription factor implicated in increasing AQP1 mRNA transcription under shear stress, and this factor is suggested as one of the cardioprotective targets of statin drugs in protecting endothelial tissue from cardiovascular disease (27). While the majority of commercially available statin drugs demonstrates an upregulatory effect on KLF2, the hydrophilic statin PravastatinTM does not have any effect on the expression of KLF2 in HUVECs (27). Considering that PravastatinTM does not have an effect on KLF2 expression, this might explain why PravastatinTM actually decreases AQP1 expression in HUVECs under shear stress *in vitro*. PravastatinTM may serve a different and novel role in HUVECs distinct from the roles of other statin drugs, and this particular role of PravastatinTM results in the ultimate regulation of AQP1. These results further demonstrate the growing amount of knowledge regarding a possible cholesterol-independent function of statin drugs in regulating the expression of AQPs. In addition, these results suggest a potential cardioprotective benefit of PravastatinTM, independent of its cholesterol-lowering function, that is related to the regulation of AQP1 in vascular endothelial cells.

The expression of PECAM-1 and BMPER were not altered by venous fluid shear stress ($p>0.05$). PECAM-1 functions as an extracellular cell adhesion molecule that allows for endothelial cell-cell adherence. PECAM-1 is also important in the overall mechanosensory complex in endothelial cells in response to mechanical stimuli, specifically shear stress (5). PECAM-1 also has potent cell-signaling properties through the downstream activation of proto-oncogene tyrosine-protein kinase Fyn (FYN) (14). This FYN kinase can internally activate the vascular endothelial growth factor receptor 2 (VEGFR2) which is another kinase crucial in many cell-signaling pathways involved in endothelial function and response to shear stress (2, 20). It is possible that the observed increase in AQP1 expression under shear stress conditions might stem from PECAM-1 dependent cell-signaling in response to an increase in shear stress. The observed decrease in AQP1 expression in HUVECs *in vitro* under shear stress conditions might stem from PravastatinTM interacting with downstream signaling components of the PECAM-1 mediated mechanosensory complex in endothelial cells. With this proposed mechanism, PravastatinTM could alter downstream effects of PECAM-1 dependent mechanosensation in endothelial cells and might regulate the downstream expression of AQP1. While PravastatinTM did not have any observed effect on PECAM-1 expression in this investigation, it is understood that another statin drug called LovastatinTM decreases the expression of PECAM-1 in endothelial cells (31). PravastatinTM is already known to be the only statin drug that does not interact with the KLF2 in endothelial cells. This might suggest that PravastatinTM functions through a different mechanism than LovastatinTM and does not directly regulate PECAM-1 expression (27).

BMPER functions as a ligand that directly interacts with the Bone Morphogenetic Protein Receptors and may play a role in endothelial cell differentiation in vasculogenesis (23). BMPER is induced by PravastatinTM under static and venous shear stress conditions ($p < 0.05$). BMPER expression was not affected by changes in shear stress ($p > 0.05$), validating the use of BMPER as a shear stress-independent control for PravastatinTM action in endothelial cells.

Since statin drugs such as PravastatinTM are considered cardioprotective agents, it is important to reconcile the advantageous function of PravastatinTM with its ability to suppress venous shear induced AQP1 expression in HUVECs. A common concern in the pathophysiology of cardiovascular disease, such as intimal hyperplasia, is vascular inflammation caused by an increase in endothelial permeability (32). When the endothelial cell layer becomes more permeable, the smooth muscle layer located beneath the endothelial layer can be exposed directly to the blood flowing through the vessel. This new environment can cause adverse changes to the smooth muscle layer resulting in vessel disease such as IH (6). In AQP1-null mutants, it has been discovered that individuals deficient in AQP1 have a decreased vascular permeability in the vessels of the pulmonary vasculature (19). While this investigation demonstrated that AQP1 is a determinant of vascular permeability in the lungs, it is possible that AQP1 functions in the vascular endothelium in a similar manner.

Aquaporin 1 might be responsible for increasing the vascular permeability that results in vessel disease. As AQP1 protein expression increases in an environment of venous flow, more water channels can function in the membrane to facilitate water movement. With an increase in water flux, it is possible that endothelial cells could swell

resulting in larger junctions between other endothelial cells. As these junctions increase in size, the permeability of the endothelial cell layer as a whole could increase. This increase in endothelial layer permeability could potentially result in downstream vessel disease and IH. Since Pravastatin™ represses the expression of AQP1 under venous flow, it is possible that the cardioprotective function of Pravastatin™ in lowering endothelial permeability functions through suppressing AQP1 protein expression. As AQP1 protein expression is repressed, the overall volume of water flux in the endothelial cells could decrease causing the cells not to swell and the junctions between cells to remain smaller and less permeable. This repression of AQP1 protein expression under venous flow conditions by Pravastatin™ could demonstrate a novel mechanism of action for statin drugs in reducing vascular permeability and inflammation.

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Chapter 3: Reflection

It was the summer of 2013. As a freshly-minted high school graduate and a future Flyer, I was ready to embark on the next stage of my academic journey. With a recent interest in the possibility of medical school after college, but with no idea what being a “pre-medical” student might mean, I desperately needed consultation on how I was even going to attempt to proceed with this ambition. As I returned home from a day of work as a cashier at the local health foods grocery store, my father informed me that he had been able to set up a meeting on campus for the following week for me to talk about selecting a major. The meeting was to be with Dr. Carissa Krane, Professor of Biology, who was then also the Associate Director of the University Honors Program. Little did I know that that meeting would change my whole college career and indeed my entire life trajectory.

I arrived on campus that next week ready to meet with Dr. Krane. My father escorted me to her office in the University Honors Program. As we walked into Alumni Hall and made our way to Dr. Krane’s office, my father coached me on how properly to introduce myself. I pretended to listen, but I was too anxious for the meeting to pay much attention to what he was saying. When we arrived at the office, and with some measure of his preparation and my inattentiveness to it, I nervously reached my hand out and said, “Good afternoon Dr. Krane. It’s a pleasure to meet you.” That was a very interesting meeting. Dr. Krane asked me a series of probing questions, not just about what interested me but how I liked to think, what kinds of questions interested me, and what kind of work gave me pleasure. At the time, I was between engineering and biology in my thinking about a possible major. Yet from our wide-ranging conversation, Dr. Krane was able to convince me that studying biology would be the most appropriate course of study

for me. Soon afterward she also showed me her laboratory in the Science Center. I remember meeting the members of the lab—Kyle McGrail, Connor Ratycz, and Mark Hawk—and listening to them discuss their research. I found myself so amazed that undergraduates were performing such complex research that I immediately began to develop the idea that I too wanted to participate in research during my time at the university. This proved to be good thinking, for as the first year began I soon came to learn that to be a Type-A, high-achieving pre-medical student, I would have to get involved in undergraduate research to improve my medical school application. So I reached out to Dr. Krane to see if she would have an availability in her lab the summer after freshman year. To my surprise and delight she had a position and I began work that summer in her lab.

Lab work was all I really thought this engagement with Dr. Krane's lab would ever be in my life. I never would have imagined that over the course of the next four years that work would take me to the opportunities of being an American Physiological Society Undergraduate Research Fellow, a Goldwater Scholar, a graduating senior with an honors thesis from Dr. Krane's laboratory, and now an accepted student at a fully-funded M.D./Ph.D. program at the University of Pittsburgh. Since I was given that opportunity to join Dr. Krane's laboratory, I have never looked back. Dr. Krane started me on my college path and she provided the foundation for my opportunities here at UD and into the future as I approach medical school and my anticipated career as a physician scientist.

While I currently write in high regard of my experiences in Dr. Krane's laboratory and where those experiences have taken me, this journey has not been without its

challenges. When I began my lab work, I was a naïve, introductory biology student who only had a very general idea about going to medical school and no real knowledge of what that really meant. I did not know what a physician really did except for what I had seen by visiting my own pediatrician. All I knew was that I wanted to help people suffering from diseases in some way, and this desire was personal. When I was a small boy, my paternal grandfather died of a rare form of stomach cancer, and in my junior year of high school, I lost my maternal grandmother to aggressive neurodegeneration related to Parkinson's disease. During those times, especially as my grandmother was dying, I felt completely useless as I could only stand idly by as her disease manifested itself. Wanting to learn and to be able to do something for others that I could not do for my grandfather and grandmother was then my first (and only) inspiration for envisioning a career in healthcare at the beginning of my college years.

I continued in the lab that summer after my first year of college, learning basic laboratory techniques and becoming familiar with the literature of aquaporins and cardiovascular biology, the eventual subjects of my Honors thesis. In this work, I shadowed Kyle McGrail, a graduate student in the laboratory. I never really spent more than a few hours a day in the lab, and I was actually rather bored by the monotony of the tasks we performed. I was frustrated by how long it took to perform lab protocols and how complex the material was. As I was having this negative reaction to laboratory work, I became all the more confident that I wanted to be a physician while developing a contrary certainty that I did not want to have a career in research. However, with a potential letter of recommendation from Dr. Krane hanging in the balance, and that requiring nothing short of my continuation in the lab, I persisted through the summer.

Frankly, because I enjoyed working with and for Dr. Krane, and I admired her knowledge and professionalism, that enjoyment and respect for her kept me going. That was a good decision, for that next school year, my sophomore year, Dr. Krane encouraged me to think about further work in the lab and worked with me to formulate an application for the 2015 American Physiological Society (APS) Summer Undergraduate Research Fellowship. If I were to get that fellowship, it would allow for me to stay on campus during the following summer and continue my work in the laboratory. Even though I had sworn off a career in the sciences the previous summer, I was excited to apply for this fellowship and work on my own project. To my surprise and delight, I was awarded the APS fellowship in late March, 2015. I never thought I would win this award, and the boost of external validation that it provided greatly aided in creating within me a new excitement around the idea of working in the laboratory again.

From Kyle, and through his remarkable generosity in sharing information, I learned how to troubleshoot my experiments and enjoy my time—all those hours—in our basement laboratory. Yet however much I learned from Kyle and benefitted from his mentoring, I was also eager to come out from behind his shadow and begin working on my own. The APS fellowship promised this possibility. I was not aware at the time that the summer of 2015 would serve as the most challenging and formative period of my growth in the laboratory.

The summer began quite differently from the previous summer. For one thing, there was no more playing around with the pipettes and empty cell culture flasks. Since the APS was paying me to undertake and further my own research, I needed to do some work that I could present at the following year's international Experimental Biology

meeting. With a fire set beneath me, I began pushing through the summer, immersing myself deeper in the scientific literature and learning more about complicated laboratory techniques. I began to appreciate how important were the skills of troubleshooting and patience in the lab as I struggled for weeks with mastering effective RNA isolation. So challenging was this that there were moments when I would break down in frustration at the continual failures of my experiments. At times that summer I even wanted to quit lab work forever, yet there was something inside me that wouldn't let me do so. I don't know if that was my pride or my naivety, but I am thankful that I stuck with the lab throughout that difficult summer and for the remainder of my undergraduate education.

I have found lab work to be difficult but immensely rewarding. There have been moments when every experiment seemed to be failing and every draft of my Honors thesis might be returned with a daunting amount of corrections and revisions to address. Yet when I was interviewing at medical schools and was asked about my greatest achievements, I often responded by talking about those very failures in my experiments and the challenges I encountered in researching and writing my thesis. While I originally thought that failures would be the death of my experiments and my enjoyment in the laboratory, I soon discovered that I found excitement in troubleshooting experiments and learning how to analyze my experiments and my ideas critically. I also discovered that I often learned far more about research through my failures than through my successes. In addition, through learning to fail and responding to and learning from those failures methodically, I developed more of a "thick skin" against failure and criticism. The very essence of scientific investigation and discovery is using experimental evidence and data to support an argument. As with all arguments, there is always an opposition that will tell

you that you are wrong and your thoughts are invalid. Before joining the Krane laboratory, I tended to take all criticism as a personal criticism of my character, ability, and intelligence. After an extended period of having to defend my research findings at local, regional, and international conferences, not to mention in regular meetings with Dr. Krane and in contacts with other faculty and students in the Department of Biology, I came to realize that criticism and questioning make up the natural, indeed the essential process of scientific research. Coming into this realization has made me a stronger scientist and a more thoughtful and humble person.

Before I joined the lab, I could barely determine the difference between a dependent and independent variable; now I feel confident in my ability to design and implement an experiment from start to finish. The lab taught me how to think as an individual and how to translate my abstract curiosities in science into tangible and testable hypotheses. Above all, when I began in the lab, I had no interest in continuing my course of research into a period of clinical training as a medical student. Now I stand ready to matriculate into an M.D./Ph.D. program in which laboratory research will be a mainstay of my education as well as a likely central feature of my future career.

In addition to my research and my scientific learning in and through the Krane laboratory, the University of Dayton has provided me more than ample resources and opportunities to fulfill my interest in clinical medicine and my related passion for community service. As a biology major, I have been able to serve my community by volunteering at the local free medical clinic, and this experience has expanded my passion for and understanding of the health care profession. Working at that clinic also taught me the value of providing medical care to underserved people.

I am wholly satisfied that I chose biology as my major, and I am very grateful for the experiences I have had in class and in the laboratory that have solidified and expanded my interests in biology and medicine. Through these experiences in and beyond the classroom, I have learned how to develop as a leader and how to serve as a mentor to other students. Being so new to science myself just four years ago, I never thought that I would develop such strong mentoring relationships with younger undergraduates. I also could not have predicted how much I would enjoy teaching some of the same skills and lessons I learned from others to young undergraduates and aspiring researchers. I am proud of and very satisfied in the decision I made four years ago to come to the University of Dayton and major in biology. I cannot imagine being at a better institution than UD for learning biology, studying broadly across the whole of the liberal arts and sciences, and developing into a budding scientist and, quite frankly, a better and more thoughtful human being.